Cloning, sequencing and sequence analysis of a chitinase gene from the secondary endosymbiont of *Glossina morsitans morsitans*

A step towards pseudo-transgenic tsetse

by

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A Thesis submitted for the Degree of Doctor of Philosophy at the University of Bristol

1998

Summary

The S-symbionts of *Glossina* spp. are pleomorphic Gram-negative bacteria that have been associated with the susceptibility of tsetse to infection with pathogenic trypanosomes via the action of chitinase. However, very little is known about the chitinase activity of this bacterium.

Secondary endosymbionts (S-symbionts) isolated from *Glossina morsitans morsitans* were tested for growth on agar plates. They were found growing in cracks formed in the surface of the agar. The organism grew just below the surface of agar deeps characteristic of microaerophiles. The S-symbiont was subsequently grown on blood agar plates under reduced oxygen conditions. The S-symbionts were transformed with a colE1 based vector pGem3Z by divalent cation treatment followed by heat shock but at very low efficiency.

Preliminary characterisation of the chitinase activity of this bacterium was achieved using chromogenic and fluorogenic soluble chito-oligosaccharides. Whole cell extracts of S-symbiont isolated from *G. m. morsitans* displayed N-acetylglucosaminidase, endochitinase and exochitinase activities. The N-acetylglucosaminidase activity displayed optimum activity between pH 6.5 and pH 7. The endochitinase and exochitinase activities had a broad pH optimum from pH 5-7.5. SDS-PAGE analysis of endo and exochitinases in cell extracts of S-symbionts isolated from three species of *Glossina* revealed that they all had an identical profile of activity found in eight bands between 39 - 130 kDa.

The chitinase gene responsible for the endo and exochitinase activity of the S-symbiont isolated from *G. m. morsitans* was cloned and sequenced. Sequence analysis revealed an open frame of 2085 bp encoding a predicted protein with a theoretical molecular weight of 76kDa and a theoretical pI of 4.78. The predicted protein showed homology to a number of chitinases belonging to family 18 of the glycosyl hydrolases. Greatest homology was found with *Aeromonas caviae* ChiA.

SDS-PAGE analysis of *E. coli* DH5α cell extracts harbouring a plasmid containing the S-symbiont chitinase gene displayed an identical profile of chitinase activity to the source organism. Boiling of these samples for 5 minutes prior to SDS-PAGE analysis left only one band of chitinase activity of size 75 kDa very close to the predicted 76kDa expected from sequence analysis.

Attempts were made to clone an N-acetylglucosamine sugar transporter gene from S-symbiont genomic DNA. PCR amplification using degenerate primers designed from conserved motifs, screening S-symbiont genomic DNA for sequences similar to *E. coli* enzyme II^{GlcNac} gene and mutant complementation approaches all met without success.

Acknowledgements

I would like to thank the following people without whose help the compilation of this thesis would not have been possible.

Dr Ian Maudlin and Susan Welburn my supervisors before they moved to Glasgow who gave me the opportunity to start this study.

To Colin Dale who initially grew the S-symbiont in agar deeps and showed it to be microaerophillic.

To my surrogate PhD advisors Lynda Moore and Peter Bennett who supported me with encouragement and kindness.

To Christine Harbour for help and guidance with the biochemical tests.

Special thanks must go to Dr Tim Walsh whose friendship and encouragement kept me sane in the darker days of this project.

To the B Floor staff, Richard, Derek, Helen, John, Gloria, Valerie, Pam *et al.* for their continual help with equipment and media.

To Cliff whose cheery disposition could light up the darkest day.

Thanks must go especially to my close friends and work mates on C and D floors and also to Prof Tim Hirst.

Especial thanks are given to my loving parents June, David, Ernest and Ann for their unconditional love and support.

Lastly to my girls, my wife Phillippa and children Lucy, Evie and Noah whose comfort and support make life.

Declaration

I declare that this thesis represents my own unaided work except where otherwise acknowledged

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January 1998

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List of Abbreviations

CIP	Calf intestinal phosphatase
DFMO	2-Difluoromethylornithine
DMSO	Di-methyl sulphoxide
EDTA	Ethylene diamine tetra acetic acid
EMBL	European Molecular Biology Laboratories
ERE	Extrachromosomal replicating elements
EtBr	Ethidium bromide
GlcNAc	N-Acetylglucosamine
$(GlcNAc)_2$	Chitobiose
Hpr	Histidine containing protein
IPTG	Iso-propyl-1-thio-β-D-galactoside
IR	Inverted repeat
Km	Kanamycin
MCS	Multiple cloning site
4MUGlcNAc	4-Methyl umbelliferyl N-acetyl-β-D-glucosamine
$4MU(GlcNAc)_2$	4-Methyl umbelliferyl-β-D-N,N'-diacetylchitobiose
$4MU(GlcNAc)_3$	4-Methyl umbelliferyl-β-D-N,N'N'' triacetylchitotriose
4MU(GlcNAc)4	4-Methyl-umbeliferyl--β-D-N,N'N''N'''-tetraacetylchitotetraose
Mr	Molecular mass
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PEP	Phosphoenol-pyruvate
PCR	Polymerase chain reaction
PNPGlcNAc	p-Nitrophenyl N-acetyl-β-D-glucosamine
$PNP(GlcNAc)_2$	p-Nitrophenyl-β-D-N,N'-diacetylchitobiose
$PNP(GlcNAc)_3$	p-Nitrophenyl-β-D-N,N'N'' triacetylchitotriose
PTS	Phospho transferase system
RBS	Ribosome binding site
RCF	Relative centrifugal force (in g's)
RNA	Ribose nucleic acid
SDS	Sodium dodecyl sulphate
SSC	Sodium chloride/sodium citrate buffer
Stz	Streptozotocin
TBE	Tris/borate buffer
Tc	Tetracycline
TEMED	N,N,N'N''-tetra methyl ethylenediamine
V	Volume
W	Weight
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

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Section One

General Introduction

1.1 Background

1.1.1 Sleeping Sickness/ Nagana

The African trypanosomiases are a group of parasitic diseases that occur across more than a third of Africa. Trypanosomiasis in man, "sleeping sickness", is responsible for numerous epidemics causing untold misery, debilitation and mortality. In addition to this, trypanosomiasis in livestock, "nagana", is arguably the most economically important livestock disease on the continent. These diseases are caused by infection with unicellular protozoan parasites called trypanosomes which belong to the genus *Trypanosoma* within the order Kinetoplastida. Their species classification is based on morphology, geographical location, hosts they infect and, occasionally, their virulence. The two sub-species of *Trypanosoma brucei brucei* that infect humans are *Trypanosoma brucei rhodesiense* (acute infections) and *Trypanosoma brucei gambiense* (chronic infections). Two other important species that infect cattle are *Trypanosoma congolense* and *Trypanosoma vivax.*

Trypanosomes are transmitted to domestic and wild animals as well as people, by tsetse flies (genus *Glossina*) as they feed on mammalian blood. Infection by these organisms is initially in the bloodstream and lymphatic system, with trypanosomes subsequently penetrating the central nervous system to produce a comatose condition which if untreated culminates in death. The trypanosome parasites that cause disease in livestock and people also infect some wildlife species with no apparent ill effects; these animals then serve as a source or reservoir of infection for flies. Tsetse, of which *Glossina morsitans* and *Glossina palpalis* are the most economically destructive of the 23 species, are found in 36 countries covering 10 million square kilometres of Africa. Trypanosomes are usually found wherever these insects are located and together they put 50 million people at risk of contracting sleeping sickness, of which only 5-10 million have access to health care (Lyons, 1991). They also put 30% of Africa's 150 million cattle at risk of trypanosomiasis. This risk precludes farmers from keeping cattle and small ruminants in these areas, accounting for Africa's low livestock productivity (the animal protein produced per hectare on the continent of Africa is only one seventieth of that produced in Europe). The impact of trypanosomiasis is even greater than these figures suggest because many of the areas inhabited by tsetse flies are potentially the most agriculturally productive in Africa. Annual losses in meat production alone are estimated at U.S. $5 billion. This economic deprivation is exacerbated by losses in milk production, tractive power, waste products and fuel, and secondary products such as hides.

In both the tsetse fly vector and the mammalian host, trypanosomes undergo a series of transformations into different forms (Vickerman, 1985). The tsetse fly ingests trypanosomes when it feeds on an animal infected by the parasite. In the fly the parasites differentiate into different forms culminating in the metacyclic form, which is able to infect mammalian hosts. When the infected fly next feeds, these metacyclic forms are injected into the skin of the host along with tsetse saliva. In the animal the parasites differentiate into a form specially adapted to live in mammalian blood. These bloodstream forms multiply by binary fission and enter the animals lymphatic and blood circulation. As flies feed on animals infected with the parasite, the flies may take up blood containing trypanosomes, thus completing the parasite's life cycle.

1.1.2 Barriers to vaccine development

Trypanosomes manage to survive the attacks of the host's immune systems by employing a defense system known as antigenic variation. This is accomplished by selective expression of one out of an estimated 1000 variable surface glycoprotein (VSG) genes (Van de Ploeg, 1982). Approximately one in every 10^6-10^7 trypanosomes undergoes antigenic variation during a cell doubling time (Miller and Turner, 1981; Lamont *et al.*, 1986). This change of VSG composition occurs spontaneously without induction by the host's immune systems or other factors in the bloodstream of the mamalian host (Doyle *et al.*, 1980; Vickerman *et al.*, 1980). Trypanosomes which now express a new VSG are thus spared from killing by antibodies produced by the host against the major antigenic types present in the infection. The outcome is therefore wave after wave of relapsing parasitaemia since, inevitably, the host is always one step behind the variable trypanosomes. Since trypanosomes have such a large repetoire of VSG genes, it therefore appears unlikely that a conventional vaccine, which primes an animal's immune system against just one or a few antigens, will be broadly effective if based on the trypanosomes variant surface proteins.

1.1.3 Control Methods

With present technology the only apparent way to eradicate trypanosomiasis would be to eradicate the vector using insecticides. However, the large short term cost of such a venture (traditional insecticide-based control problems often have a price tag that is larger than the budget of the ministry of health of those countries that have the greatest need for such programs), together with the environmental damage caused by the insecticide weigh heavily against such a strategy. It could also be questioned whether

this would actually be possible since tsetse are likely to develop resistance. Therefore, since eradication of the vector is not feasible, the long term approach to trypanosomiasis is towards control and not eradication.

A number of different control measures have been practised over the last 80 years. The most basic of these measures involves keeping people and animals out of high risk areas. This method is still being practised by the Massai tribe of east Africa, who keep their cattle out of areas that they know are tsetse infested. Other methods are either aimed at the insect vector or directly at the trypanosome. Control methods aimed against the tsetse vector involve the use of selective ground spraying with residual insecticides, and aerial spraying of non-residual insecticides; but, application of such compounds over large areas is discouraged because of the pollution this causes to the environment. Traps and targets strategically placed in fly habitats have proven to be efficient in reducing fly populations (Vale, 1985). These targets and traps have greatly improved over the last few years, and in some areas their deployment has reduced fly populations to tolerably low levels (Lancien, 1993). However, this control method has been demonstrated to work only in some types of areas and only for some species of tsetse. Other methods include the treatment of livestock with insecticide, and the clearing of vegetation in an attempt to remove the habitats of the flies, the latter being an unacceptable waste of diminishing natural resources. In terms of control measures aimed directly at the parasitic trypanosome, prophylactic and curative drug treatments are the most widely used. The drugs available are effective but also relatively expensive (a full course of treatment with melarsoprol costs US $200 and with DFMO US $500 per patient; Williams *et al.*, 1993). Although the drugs used to treat livestock are cheaper some are suspect mutagens and even the ones used to treat people have toxic and sometimes lethal side effects (Van-Nieuwenhove, 1992). Moreover, no new commercial drug has been introduced for the treatment of animal trypanosomiasis in the last 30 years (Murray *et al.*, 1991) and DFMO, approved for use in 1990, was the first new drug to treat sleeping sickness to be introduced for 40 years (Trypanosomiasis Disease Research news, 1990). Widespread use of the few drug compounds available has also led to increasing parasite resistance.

An additional control method is to stock cattle of a few ancient African breeds, such as the N'Dama and the west African Shorthorn which have an innate tolerance to trypanosome infection. The genetic ability to resist pathogenic effects of infection is called trypanotolerance. Use of trypanotolerant cattle is perhaps the most attractive of the control methods available and has made livestock rearing possible in tsetse infected

areas, but this method also has its drawbacks. Firstly, the degree of the disease resistance in an animal is not absolute; levels of trypanotolerance are reduced in animals under stress, particularly those with poor nutrition, a common condition of livestock raised in Africa's marginal farming areas. Secondly, although meat and milk production compares favourably with other breeds, trypanotolerant animals raised in traditional farming systems are typically small and are therefore not ideal for draught work. Finally and most importantly, few such animals are available. In spite of their importance in tsetse infected areas where other livestock cannot survive, trypanotolerant cattle constitute only 5% of the cattle raised where tsetse flies occur. Numbers of such cattle are rising, but slowly. Merely to double the number of trypanotolerant cattle alive today will take 15 years, at present-day breeding rates.

The only genetically based control program that has so far been introduced against tsetse is the release of sterile male tsetse (Takken *et al.*, 1986) to "dilute out" the infected tsetse. Other such genetically based control programs have had some success in the past; the use of sterile male release for the control of the new world cattle screw worm fly, *Cochliomyia hominivorax*, integrated with other control measures, served to eradicate the insect from southern USA, Mexico (Krasfur *et al.*, 1986) and Libya (Lindquist *et al.*, 1992). However, in contrast to the cattle screw worm fly, where every fly can cause disease, the low infection rate of tsetse with trypanosomes (typically <5%) means that large release numbers would be required to have a significant effect on disease transmission. Further to this, this type of approach to control is labour intensive and hence very expensive.

1.1.4 Genetic manipulation of insects

Recombinant DNA technology enables manipulation of genetic material *in vitro* and its integration into the genomes of a variety of host species. This was not possible with the classical genetic crossing techniques, without causing massive genetic rearrangements. The advent of recombinant DNA technology offers not only the possibility of producing transgenic strains of insects that are incapable of transmitting disease, but also of altering the genetic structure of insect populations (Crampton *et al.*, 1990; Handler and O'Brochta, 1991; Miller, 1992; James, 1992; Aldhous, 1993 and Richards, 1993) The combination of genetically manipulated refractory insects, together with an efficient drive mechanism, could then serve as a control method to stem the spread of trypanosomiasis in Africa. This genetic control method is likely to have numerous advantages over current control methods since modifications to the genome would be

inherited by subsequent generations and therefore be self-supporting and sustainable. Such genetic control methods are likely to be inexpensive to support in the field once the initial research has been accomplished, and they have the advantage that being maintainance free they are not subject to breakdown with resultant epidemics through political upheavals (Lyons, 1991).

This genetic control strategy depends on progress in three different areas:-

(1) The development of efficient mechanisms for introducing recombinant DNA constructs into vector genomes,

(2) The identification of parasite inhibiting genes,

(3) The harnessing of mechanisms for moving these genes into natural populations.

Most research to date has focused on producing transgenic mosquitoes that are medically important in that they transmit a number of infectious agents, including *Plasmodium* species responsible for malaria (causing > 1 million clinical cases each year in Africa alone), arboviruses responsible for diseases such as yellow fever, dengue, dengue haemorrhagic fever, Japanese encephalitus and La crosse fever, as well as nematode worms responsible for elephantiasis and filariasis.

At present, however, there are some serious problems to overcome before producing such medically useful transgenic insects. One large problem is that although there are a number of mechanisms available for introducing foreign DNA into insect cells and embryos (use of calcium phosphate precipitation (Wigler *et al.*, 1977), dextran sulphate (Lopata *et al.*, 1984) polybrene (Durbin and Fallon, 1985), electroporation (Chu *et al.*, 1987), lipofection (Felgner *et al.*, 1987) and micro-injection, there is a distinct lack of suitable transformation vectors for these insects.

Transformation systems are available at present for only one insect, the fruit fly *Drosophila*, which is not an important disease vector. This transformation system is based on a transposable element, the *P* element, which was initially isolated due to the genetic defects which resulted from induction of its mobility in inter strain crosses of *Drosophila melanogaster* (Kidwell *et al.*, 1977).

The demonstration of *P* element transposition in distantly related drosophilids (Brennan *et al.*, 1984; Daniels *et al.*, 1985) indicated that *P* element mobility might not be phylogenetically restricted. This factor prompted researcher workers to try to manipulate the mosquito genome using *P* element-based transformation systems.

Although there have been several reports of the sucessful integration of DNA into the genome of three mosquito species (Miller *et al.*, 1987; McGrane *et al.*, 1988; Morris *et al.*, 1989), none of these integration events were *P* element mediated; instead they were due to random integration events or recombination between vector sequences and the host chromosomes.

The failure to recover germline transformants in heterologous systems by direct application of the *D. melanogaster P* element systems has prompted research into the precise mechanism of *P* transposition and may ultimately identify the genetic factors involved in the inability of the *P* element to transpose in non-drosophilids. Presumably these could then be incorporated into *P* constructs to form the universal vectors originally envisaged. However, *P* elements may never function as efficient transposition mediated transformation vectors in non-drosophilids.

Research is also focusing on isolating endogenous transposable elements from specific vector species which could be engineered to produce suitable transformation vectors and on other systems such as retrotransposons or site specific recombination systems (Sadowski 1986, Golic and Lindquist 1989; Morris *et al.*, 1991). Likely candidates are the mariner and minos transposable elements (Miller, 1992; Haymer and Marsh, 1986). The Ac and Spm elements of maize and the Tcl element from nematodes have also been suggested for prospective transformation vectors by virtue of their transposition in a number of diverse organisms.

1.1.5 Drive mechanisms

In addition to the transformation of insects producing individuals that are incapable of transmitting disease, there is also a need to "drive" the refractory phenotype through wild type populations of insects if this approach is going to be useful as a control method in the field. Two promising drive mechanisms are meiotic drive and cytoplasmic incompatibility. Meiotic drive refers to cases where a given chromosome is transmitted to more than 50% of offspring and any desirable gene linked to the driven chromosome would therefore eventually reach fixation. Meiotic drive can be associated with *P* element transposition. In suitable crosses the *P* element can spread rapidly in the germline to all chromosomes so that the *P* element is present in all offspring. The combination of the rapid spread of the *P* element in fruit fly populations together with the passive transport of *Drosophila* around the world with the international fruit trade

has been implicated for rapid worldwide invasion of *P* elements (Kidwell *et al*, 1983). Flies captured in the wild before 1950 did not carry these elements. In contrast every natural population of *D. melanogaster* that has been sampled since 1978 has been found to carry multiple copies of P elements (Anxolabehere *et al.*, 1988). In the case of the *P* element, once the transposon has moved through a population there is a reduction in transposition. This is in part due to to the formation of a number of truncated nonautonomous elements that produce a defective transposase inhibiting transposition (Rio, 1991). The *P* element model would therefore suggest that once a transposable element has spread through a population it is of no further use for genetic engineering within that population. The choice of a transposable element to drive a refractory gene will therefore need to be based on its initial absence in the recipient population at least for transposable elements like the *P* element (Kidwell and Ribero, 1992).

An alternative mechanism, cytoplasmic incompatibility, shows great promise for use as a means of driving disease refractory genes through insect populations. Cytoplasmic incompatibility was first characterised in the mosquito *Culex pipiens*, (Barr, 1966; Laven, 1967) and has since been identified among a diverse array of insect species spanning five orders (Stevens and Wade, 1990). The phenomenon of cytoplasmic incompatibility is associated with the Rickettsia-like organism *Wolbachia pipientis* which infects insect gonadal tissue and is generally transmitted maternally. Infection with this bacterium gives a breeding advantage to females; in most cases, infected females produce infected male and female offspring regardless of whether the mated male was infected or not. However progeny viability is reduced when an infected male mates with an uninfected female. This reduction in numbers of offspring varies from complete in some hosts to partial or no reduction in others (Holden *et al.*, 1993), but few if any hatch in the majority of species. Reciprocal crosses and crosses within an infected strain are normally fully compatible (Yen and Barr, 1973; O'Neill and Karr, 1990; Brewer and Werren, 1990). The cellular basis of cytoplasmic incompatibility is due to defects in the structure and/or function of the sperm during fertilisation. The presence of the micro-organism seems to modify the sperm so that they are no longer compatible with an uninfected female's eggs, causing many embryos to die. This effect allows the microbe to spread rapidly since infected females can mate with any male and produce viable offspring, whereas crosses between uninfected females and infected males produce few if any offspring. The presence of the parasite decreases slightly the number of eggs that host females produce, but otherwise has no known ill effect. The spread of this maternally inherited organism through a previously

uninfected *Drosophila simulans* population in California has been estimated at spreading in excess of 100km per year (Turelli and Hoffman, 1991). The ability of natural *Wolbachia* infection to spread through populations of insects could be manipulated to spread other maternally inherited organelles or symbionts at the same time. Another of the potential strengths of cytoplasmic incompatibility as a drive mechanism derives from the observation that non-reciprocal incompatibility can also occur between infected populations. This effectively mimics the effects seen between infected and uninfected individuals which presents the possibility of repeated cytoplasmic sweeps through a given population.

1.1.6 Pseudo-transgenic Tsetse

The difficulties encountered in transforming the mosquito genome are amplified when attempting to transform the tsetse fly. Not only are there no vectors available for tsetse fly transformation, but the fact that tsetse give birth to live larvae means that micro-injection of eggs would be too invasive and therefore impractical. In addition to this, little information is available regarding the tsetse genome and no linkage maps are available. However, the organisms responsible for cytoplasmic incompatibility have been detected in the gonadal tissues of certain species of tsetse flies (Beard *et al.*, 1993b). In addition to this, tsetse harbour midgut symbiotic bacteria called secondary symbionts (S-symbionts) that are maternally inherited. The *Wolbachia* infections in the gonadal tissues of the tsetse could therefore provide the impetus to drive these midgut symbionts through tsetse populations. Moreover, the presence of these S-symbiotic bacteria presents the possibility of developing a system that uses them as vehicles for expressing genes in tsetse that reduce their vector competence. This system would avoid the barriers imposed by transforming the tsetse genome and provide a "short cut" to the production of refractory insects. A similar pseudo-transgenic approach has been envisaged to produce refractoriness in the triatomine bug, *Rhodnius prolixus*, to infection with *Trypanosoma cruzi* (Beard *et al.*, 1992). The symbiont *Rhodococcus rhodnii* has already been transformed using a shuttle plasmid that has shown stable inheritance *in vitro* and *in vivo* (Beard *et al.*, 1992), suggesting that maintainance of transformed symbionts in insects may be possible for extended periods of time, with little or no selection. The important parameters involved in the production of pseudo-transgenic insects and tsetse in particular are the transformation efficiency, infection rate and plasmid stability in the bacterial symbionts. Since tsetse S-symbionts carry a number of endogenous plasmids that are stabily inherited, if an exogenous plasmid is

not stable then an endogenous plasmid could be engineered to carry the refractory gene using a transposon based transformation vector. The bacterial S-symbiont of the tsetse fly has also been implicated in production of susceptibility of the fly to infection with trypanosomes via production of the enzyme chitinase (see section 1.13.5). The wealth of information regarding bacterial transformation, together with the fact that the bacterial S-symbiont is maternally inherited and its involvement in susceptibility to infection, suggest that this pseudo-transgenic system could well be manipulated to produce future populations of pseudo-transgenic tsetse that are refractory to infection with trypanosomes. This thesis focuses on the chitinase system of the bacterial S-symbiont of the tsetse fly and its proposed involvement in rendering tsetse susceptible to infection with trypanosomes. The current literature involving bacterial chitinase systems and the cloning and analysis of their respective genes is therefore reviewed, as well as the involvement of chitinases in the transmision of other vector borne parasites and diseases.

1. 2 Chitin

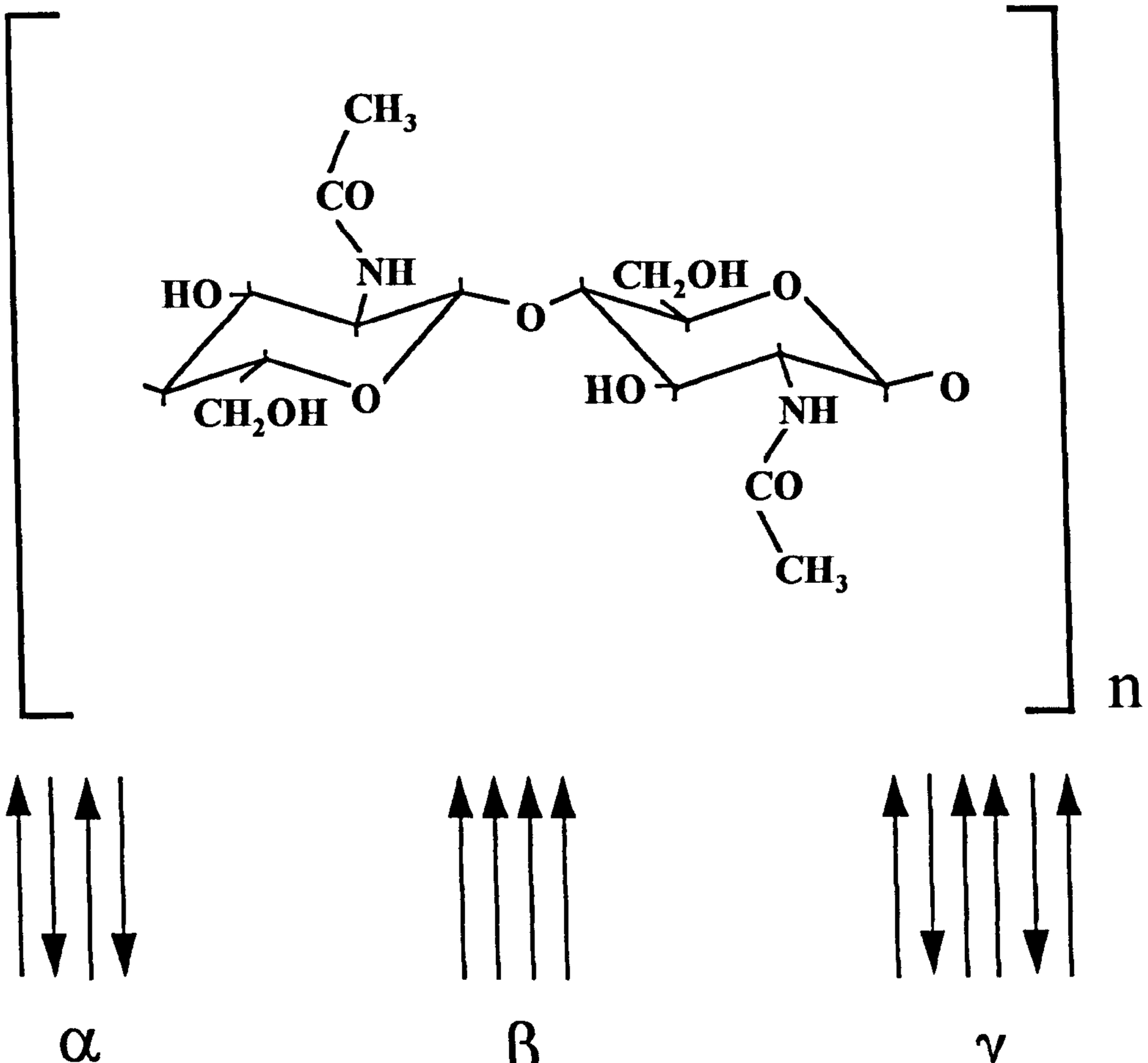
Chitin is one of the most abundant substances of biological origin on our planet. It is a fibrous polysacharide, consisting of linear chains of β1-4 linked molecules of N-acetyl glucosamine, although glucosamine has also been found to varying degrees (Neville, 1975; Kramer and Koga, 1986). Chains of this homopolymer associate with one another by very strong hydrogen bonding between N-H and C=O groups of adjacent chains (Cabib, 1987). The hydrogen bonding accounts for the great insolubility of chitin in water and for the formation of fibrils. The orientation of the microfibrillar chains allows for three polymorphic forms,α,β or γ chitin (figure 1.1). α chitin is the most stable and common form, found in arthropods and fungi, and is composed of an antiparallel arrangement of adjacent chains. β chitin is composed of parallel chains, and γ chitin is composed of repeating layers of two parallel chains and one antiparallel chain. The different forms of chitin give rise to different mechanical and biophysical properties

The abundance of chitin in nature is due to the fact that it is a common constituent of insect exoskeletons, fungal cell walls, and the shells of crustaceans, as well as being found in nematodes, molluscs, worms, certain diatom algae (Cohen, 1987) and certain protozoa (Schlein *et al,* 1991). It is, however, completely absent from vertebrates and *sensu stricto* from plants, although polymers rich in (1-4)-β linked N-acetylglucosamine

have been reported (Gooday, 1994)

In filamentous fungi, chitin is very often the major component of the cell wall, being cross-linked with other structural components, eg β-glucans (Gooday, 1994). In the shells of crustaceans, chitin fibrils are embedded in a matrix of calcium carbonate, phosphate and protein (Muzzarelli, 1977). In insects and other invertebrates, chitin microfibrils are found linked with various proteins by covalent and non covalent bonding to produce ordered structures. This chitin-protein lattice confers the needed rigidity to structures such as the cuticle and the peritrophic membrane of insects (Hepburn, 1985), as well as providing the first line of defence against adverse surroundings and attack by micro-organisms and parasites. The importance chitin plays in nature and its widespread occurrence has caused it to be the subject of many studies. (see Kramer and Koga, 1986 and Cohen, 1987 for further information on invertebrate chitin).

Figure 1.1 Repeating unit of chitin, and the arrangement of adjacent chains in α, β and γ chitin (adapted from Cabib, 1987).



1. 3 Chitinolysis

Chitinolysis is a term used to describe the breakdown of chitin solely by hydrolysis of glycosidic bonds (Gooday, 1994). The enormous amounts of chitin produced continuously in nature need to be recycled on a massive scale. This is mostly accomplished by chitinolytic bacteria which produce enzymes (chitinases) that are capable of hydrolysing the β 1-4 linkage between two consecutive N-acetyl glucosamines in chitin. Chitinases, however, are not restricted to bacteria, and are found in all organisms capable of producing chitin including fungi and invertebrates (mainly nematodes, insects and crustaceans), as well as in many other organisms that do not produce chitin including a wide variety of higher plants and all classes of vertebrates (Cohen, 1987).

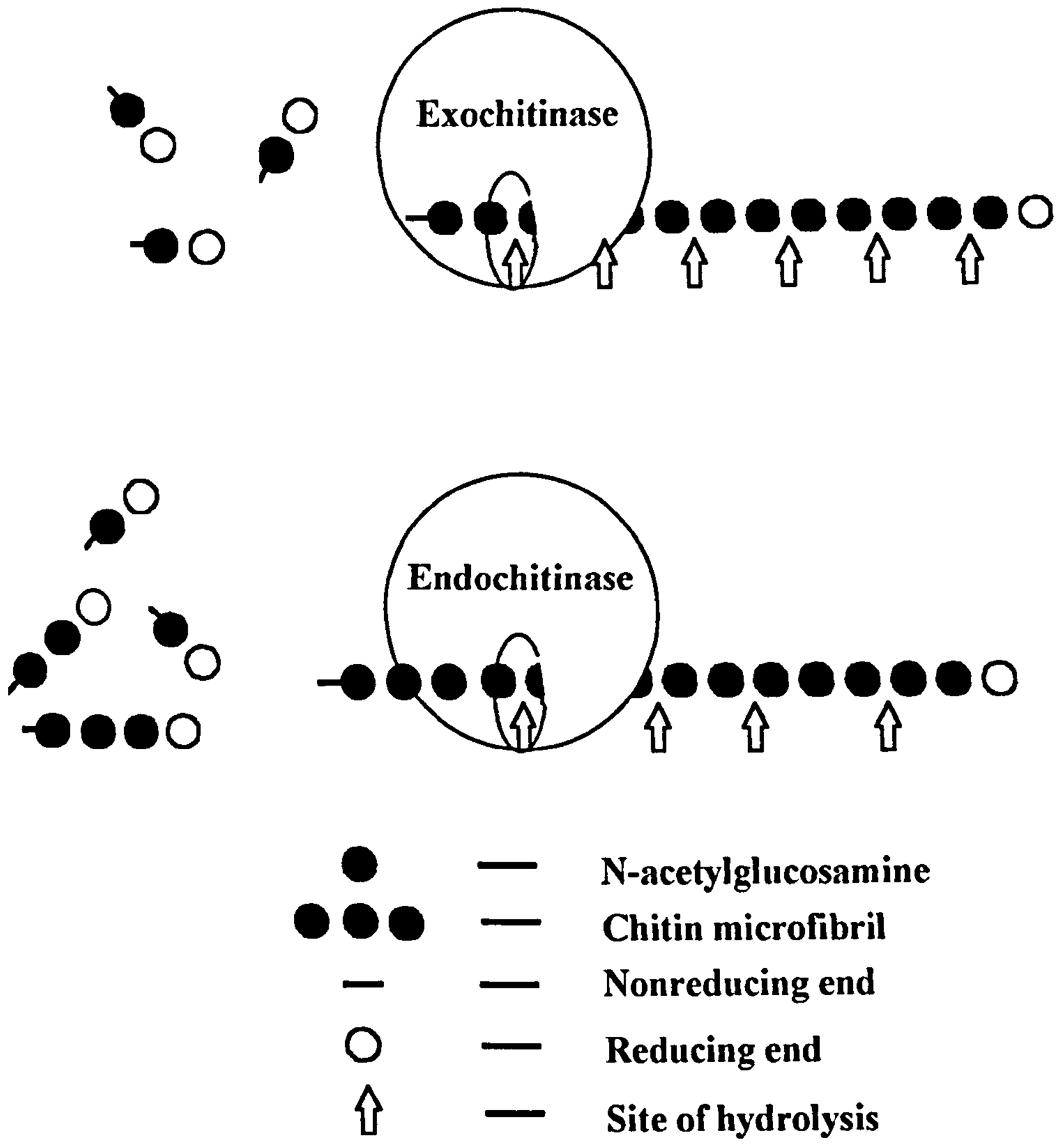
Bacteria produce chitinases primarily to utilise chitin as carbon, energy and nitrogen sources (Gooday, 1990; Chen *et al.*, 1991; McCreath and Gooday, 1992). The function of chitinase in plants is considered to be defensive as part of a pathogen related response

including other defensive molecules such as phytoalexins, proteinase inhibitors, ribosome inactivating proteins and other lytic enzymes (Hart *et al.*, 1993). Chitinases are potent inhibitors of fungal growth, and plants produce them both constitutively and in response to microbial infection and some injuries (Pegg and Young, 1982; Boller *et al.*, 1985). Purified chitinases have been shown to be able to inhibit fungal growth *in vivo* in combination with the activity of β-1,3-glucanases by causing lysis of hyphal tips (Roberts and Selitrennikoff, 1986; Broglie *et al.*, 1991), and it has also been shown that enhanced chitinase levels in transgenic plants can reduce the damage caused by pathogens (Jach *et al.*, 1992). Chitinases in fungi are thought to have autolytic, nutritional and morphogenic roles (Gooday 1994); in particular, growth at the apex of fungal hyphae has been proposed to be the result of a harmonius balance between the processes of synthesis and lysis by enzymes such as chitin synthetase and chitinase (Cabib *et al.*, 1992). In yeast, chitinase has been shown to be necessary for cell separation during growth (Kuranda and Robbins, 1991) and chitinases in crustaceans and insects are associated with the need for partial degradation of the old cuticle. The secretion of these enzymes is subject to hormonal control (Spindler-Barth, 1993). Finally, chitinases in vertebrates are usually found in the digestive tract (Perrakis *et al.*, 1994).

1. 4 Classification of chitinases

Chitinases are characterised by the specific properties of their hydrolytic activity. Endochitinases, Enzyme Commission (EC) number 3.2.1.14 (I.U.B., 1984), act randomly by splitting bonds within the chitin polymer, whereas exochitinases (EC 3.2.1.14), act progressively by releasing the N-acetyl glucosamine dimer, chitobiose, from the non reducing end of the chitin molecule (figure 1.2). β-*N*-acetylglucosaminidases, (chitobiases) (EC 3.2.1.30), are also members of the chitinase family that breakdown chitobiose to two *N*-acetylglucosamine monomers and sometimes release N-acetyl-glucosamine monomers from chitin (Flach *et al.*, 1992). These three enzymes are collectively known as the chitinolytic system (Gooday, 1990). Some plant chitinases also display a more or less pronounced lysozyme activity (EC 3.2.1.17) (Terwisscha van Scheltinga *et al.*, 1994) corresponding to the cleavage of a glycosidic bond between the C1 of *N*-acetylmuramic acid and the C4 of *N*-acetylglucosamine in bacterial peptidoglycan. Transglycosidase activities associated with exochitinase activities have also been detected, (Usai *et al.*, 1990; Takayanagi *et al.*, 1991; Flach *et al.*, 1992).

Figure 1.2 Mechanism of action of exochitinase and endochitinase (adapted from Sahai and Manocha, 1993)



1. 5 Sub-classification of chitinases

Chitinases have recently been classified as classes I and II based on multiple alignments of their amino acid sequences (Perrakis *et al.*, 1993). These classes correspond to families 19 and 18 of the glycosyl hydrolases, respectively, which were classified earlier by Henrissat (1990, 1991) and Henrissat and Bairoch (1993). Class I chitinase activity is found exclusively in the plant kingdom (Henrissat, 1991, Perrakis *et al.*, 1993) and contains chitinases that are homogenous in size and primary structure, with an average of about 300 amino acid residues (Perrakis *et al.*, 1991) (see Flach *et al.*, 1992 and Sahai and Manocha, 1993 for reviews of plant and fungal chitinases). In contrast, class II chitinases are found in plants, fungi, viruses and Gram-positive and negative-bacteria and vary in size from 290-865 amino acid residues. All class II chitinases have short regions of high homology within a region of about 200-300 amino acids, but the class as a whole displays little overall homology between its members. The relatively low

similarity scores found between members of class II are due, in part, to their different lengths (Perrakis *et al.*, 1993). Class I is further divided into two distinct subgroups. Group Ia has an approximate 40 amino acid N-terminal domain that is rich in cysteine residues and connected to the main structure by a glycine and proline rich hinge region; this region is absent in group Ib. Class II also contains a subgroup, IIa, (Perrakis *et al.*, 1993) encompassing all the eukaryotic chitinases of group II, based on the higher similarity between these enzymes compared with the rest of group II chitinases. Perrakis *et al.*, (1991) further report that it is likely that the two chitinase classes represent different enzymatic chitinase functions.

1. 6 Chitinolytic bacteria

Bacteria possessing chitinolytic systems are extremely important globally for the recycling of chitin into useable forms (Gooday, 1994). If chitin was not degraded in this manner it would become a serious strain on the carbon and nitrogen cycles. Johnstone (1908) recognised the importance of marine bacteria in this cycling early in this century, estimating that the amount of chitin produced annually by copepods alone to be several billion tons, whereas marine sediments contain little chitin. Studies earlier this century reported chitin digestion by mixed cultures of marine bacteria (Zobell and Rittenberg, 1937). Zobell and Rittenburg (1937) also record the isolation of 31 different pure cultures of marine bacteria and noted that between 0.1% and 1% of all marine bacteria were chitinolytic. They found chitinolytic bacteria in nearly all marine sediments tested. Such bacteria were most numerous in the surface layers of mud and topmost depths of the water column. Amongst Gram-positive bacteria, chitinase production is widespread in the Actinomycetes, *Streptomyces* spp., *Arthrobacter* spp. and *Nocardia* spp., and in the endospore forming genera, *Bacillus* and *Clostridium*. Gram-negative chitinolytic bacteria include members of the genera *Aeromonas*, *Alteromonas*, *Chromobacterium*, *Photobacterium*, *Pseudomonas*, *Serratia*, *Vibrio*, and the gliding bacteria *Cytophaga*, *Lysobacter* and *Chitinophaga* (Arnold, 1993). Chitinolytic activity is, however, absent from archaebacteria (Gooday, 1994). Gooday (1994) reports that the soil contains many chitinolytic bacteria including members of the genera *Pseudomonas*, *Aeromonas*, *Cytophaga johnsonae*, *Lysobacter*, *Arthrobacter*, *Bacillus* and actinomycetes. Chitinolytic bacteria are also abundant in estuarine and marine environments, characteristic genera being *Serratia*, *Chromobacterium*, *Pseudomonas*, pigmented *Cytophaga*-like bacteria, *Aeromonas*, *Streptomyces*, *Photobacterium*, *Bacillus*, *Clostridium*, *Haloanaerobacter chitinovirans*, and also in

fresh waters including *Serratia*, *Chromobacterium*, *Pseudomonas flavobacterium* and *Bacillus* with *Cytophaga johnsonae* and actinomycetes in sediments (Gooday, 1994).

1. 7 Complexities of chitin degradation

Although the chitinolytic system has previously been stated to involve the combined actions of three enzymes (see section 1.4), the degradation and utilisation of chitin is more complex than this, involving a number of inter-related sytems and many enzymes and proteins. This sophistication in chitin utilisation has been highlighted for *Vibrio furnissii* in a series of papers (Yu *et al.*, 1987 and 1991; Bassler *et al.*, 1989; 1991a and 1991b,). *Vibrio furnissii* has the ability to sense and move towards N-acetylglucosamine (GlcNAc) (Bassler *et al.*, 1989 and 1991a) and the presence of an extracellular or membrane bound chitinase can be demonstrated by zones of clearing around colonies in colloidal chitin plates. The authors suggested that extracellular chitinase initially solublises crystalline chitin, with the soluble oligosaccharide products eliciting physiological responses in *V. furnissii*, inducing the expression of a number of proteins, some soluble and others membrane-associated, including enzymes, transporters, chemoreceptors and possibly porins and periplasmic solute binding proteins (Bassler *et al.*, 1991a). The induced proteins provide *V. furnissii* with the apparatus necessary to capture and degrade chitin oligosaccharides. The soluble oligosaccharide products penetrate the outer membrane possibly via specific porins and are hydrolysed by the concerted action of a unique membrane bound endoenzyme (chitodextrinase) and a β-*N*-acetylglucosaminidase producing N-acetyl glucosamine and chitobiose as end products. The gene for the chitodextrinase has been cloned (Bassler *et al.*, 1991b) and sequenced (Keyhani and Roseman, 1996b); sequence analysis revealed an open reading frame encoding a pre-protein of 1046 amino acids with an estimated molecular weight of 112,690 Da. The chitodextrinase contained no recognisable chitin binding domains and cleaves soluble oligomers but not chitin to di- and trisaccharides. The β-*N*-acetylglucosaminidase gene has also been cloned (Bassler *et al.*, 1991b) and sequenced (Keyhani and Roseman, 1996b); the gene encodes a 69,377 Da protein of 611 amino acids that displays significant amino acid sequence homology to the α and β chains of human hexosaminidase. The β-*N*-acetylglucosaminidase has greater activity with N-acetyl glucosamine oligosaccharides $(GlcNAc)_n$, where n= 3-6 than with $(GlcNAc)_2$ and only degrades $(GlcNAc)_2$ at non-physiological pH (Keyhani and Roseman, 1996b). The $(GlcNAc)_2$ and GlcNAc end products are then further degraded

via one of two pathways:- (1) The Monosacharide pathway, whereby periplasmic GlcNAc is taken up by enzyme II ^{GlcNAc} of the PTS system and is concomitantly phosphorylated to GlcNAc-6-P as it is translocated across the plasma membrane. GlcNAc-6-P is then deaminated, deacetylated and fed into glycolysis; (2) evidence for a second pathway comes from experiments involving an enzyme II ^{GlcNAc} mutant which could grow at normal rates on $(GlcNAc)_2$ and with the presense of a cytosolic chitobiase which showed a different induction profile to the membrane bound one. (Bassler *et al.*, 1991a). The disaccharide pathway involves a $(GlcNAc)_2$ permease (Keyhani *et al.*, 1996) that is expressed by *V. furnissii*. Chitobiose is further hydrolysed to NAG by the cytoplasmic chitobiase after translocation and the GlcNAc phosphorylated by an ATP dependant kinase to GlcNAc-6-P and fed into glycolysis as in the monosaccharide pathway. In addition to the above complexities, *V. furnissii* has been demonstrated to be able to bind specifically to chitin. Yu *et al.* (1991) tested intact cells for their ability to bind to carbohydrates immobilised on gel beads. They reported the presence of a calcium requiring lectin with a broad sugar specificity, the highest affinity being for β-N-acetylglucosamine followed by β-galactosamine and α-mannose. Additionally they found that protein synthesis was required both for initiation and maintainance of specific adhesion and that these processes occurred under conditions of limiting amino acids, well below the level required for cell division. This means that expression of lectin activity is a major priority for these cells, that lectin turns over more rapidly than whole cell protein, and that the lectin is preferentially expressed since adhesion continues at nutrient levels below that required to support cell growth. Lectin synthesis and turnover appears to be a complex phenomenon, comprising a nutrient sensorium which constantly monitors the surronding environment. These speculations make teleological sense, as otherwise nutrients would be lost by dilution and water currents. *V. furnissii* attatches to the sugar substratum when conditions are favourable for at least minimal protein synthesis and remains attatched as long as the nutrients required for protein synthesis are present. When the medium is depleted the cells release and migrate towards a more favourable environment.

Bassler *et al.* (1989 and 1991a) reported that the adhesion/release apparatus is complemented by the ability of *V. furnissii* to move chemotactically towards GlcNAc. Chemotaxis towards GlcNAc was demonstrated in swarm plates and capillary assays and increased 2-3 fold when the cells were starved. Furthermore, the chemotactic response of *V. furnissii* is amongst the most potent ever reported in bacteria. Interestingly chemotaxis to GlcNAc was found to be inhibited by compounds that feed

into or are part of the Krebs TCA cycle; lactate, pyruvate, succinate and fumarate caused inhibition whereas other catabolites gave partial inhibition, had no effect or stimulated taxis. The inhibitory effects corresponded largely with the ability of the cells to oxidise the compounds; substrates that were oxidised rapidly were the most effective inhibitors, with the exception of isocitrate which was oxidised at a very rapid rate but did not inhibit taxis. The results suggest a link between catabolism and taxis in this organism, i.e. interactions or "cross-talk" between systems that are regulated by protein phosphorylation. Bassler *et al.* (1991a) speculated that the chemotactic response to GlcNAc and its oligomers functions normally when the Krebs cycle is operating at a low rate, but is inhibited when the cycle functions at a high rate. Hence chemotactic mechanisms would be switched on as the cells disassociate from chitin. These observations are consistent with the results of Zobel and Rittenberg (1937) who found that efficient solubilisation of chitin only occurred after marine bacteria had been allowed to settle upon strips of chitin.

1. 8 Adhesion and pathogenesis

Other bacteria that have been shown to adsorb to chitin are *Vibrio harveyi*, *Vibrio parahaemolyticus* and possibly *Vibrio cholerae* serovar 01. Montgomery and Kirchman (1993) demonstrated that the specific attatchment of *V. harveyi* to chitin is mediated by chitin binding proteins associated with cell membranes and they found a 53 kDa peptide to be involved in this attachment process. They also hypothesised that this bacterium may use an outer membrane chitinase to mediate attachment to chitin particles and suggested that one possible mechanism could be that part of the chitinase binds to chitin as a lectin while associated with the membrane but then it loses this binding capacity and gains enzymatic activity after processing and secretion. Watanabe *et al.* (1990b) described this sort of processing for *Bacillus circulans,* a bacterium which produces two major chitinases. One of these chitinases is the proteolytic degradation product of the other, the processed chitinase maintaining activity but losing the capacity to bind to chitin. The ability of marine bacteria to adhere to chitin has also been implicated in their pathogenic capability. Kaneko and Colwell (1973) recorded that more than 70% of cases of food poisoning in Japan are caused by injestion of seafood contaminated with *V. parahaemolyticus.* They further described strong adsorption of *V. parahaemolyticus* isolated from estuarine Chesapeake bay to chitin. The adsorption was decreased by increasing values of salinity and pH from those of estuarine to sea water, which would favour the retention of the pathogen in the estuary. Nalin *et al. (*1979) also

reported that production of chitinases by *V. cholerae* 01 suggests that pandemic strains may also have an extra human ecological niche associated with marine organisms; this hypothesis is consistent with ecological data on *V. cholerae* in Chesapeake Bay, occurrences of sero group 01 cholera linked to infection of crabs in Louisianna and culture of 01 from crabs and shrimps. Nalin *et al.*, (1979) also demonstrated adsorption of *V. cholerae* 01 onto chitin and demonstrated that this partially protected the organisms from gastric acid. Sasmal *et al.* (1992) also reported the purification of a GlcNAc specific lectin from *V. cholerae* and showed its ability to impair adhesion of *V. cholerae* to rabbit intestinal epithelial cells; they speculated that, in addition to its possible participation in adhesion of *V. cholerae* cells to rabbit epithelial cells, the lectin may enable bacteria to adhere to chitinous organisms. Huq *et al.* (1984) recorded that *V. cholerae* has the ability to bind to surfaces of copepods and that the combination of high temperature and abundance of live copepods is optimum for survival of this bacterium in the aquatic environment. This would therefore, in part, explain the epidemiology of cholera and non 01 *V. cholerae* gastroenteritus in certain geographical areas such as Bangladesh.

Adhesion of chitinolytic organisms to chitin is, however, not limited to marine organisms. *Cytophaga johnsonae*, a ubiquitous soil organism, characteristically binds to chitin as it degrades it (Gooday, 1994). Pell and Gottschal (1986) illustrated direct contact between cells of chitinolytic *Clostridium* strain 9.1 and chitin fibrils and Gilboa-Garber (1989), suggested that lectins can be lytic enzyme positioning sites, that act synergistically with enzymes. These sites are important for the efficient hydrolysis of biopolymers, and chitinolytic bacteria may use a multimolecular complex to bind and degrade chitin, analogous to cellulolytic systems (Lamed *et al.*, 1987); Anderson and Salyers, 1989)

1. 9 Utilisation of chitinolytic bacteria and chitinases for biocontrol of plant pests and pathogens

The soil contains a wide variety of organisms that interact with plants including bacteria, fungi and nematodes. Micro-organisms are especially abundant in the rhizosphere, the area that surrounds and is influenced by the plant root. Early studies by Mitchell and Alexander (1961) described naturally occurring mycolytic or fungi-lysing soil bacteria of the genera *Bacillus* and *Pseudomonas* which suppress soil F*usarium* by means of chitinase activity. Sneh (1981) also described naturally occurring chitinolytic bacteria, identified as *Arthrobacter spp* and *Serratia liquefaciens,* and reported that the introduction of a chitinolytic bacterium from the genus *Arthrobacter* into the rhizosphere protected carnation seedlings against *Fusarium* wilt. Miller and Sands (1977) further described the effect of chitinase, obtained from a commercial supplier, on certain nematodes; the chitinase was toxic to certain nematodes, in particular *Tylenchahncha dubius,* the toxicity being greater in aqueous solution than in soil. Previous reports have also shown that the addition of chitin to soil increased populations of chitinolytic bacteria, especially actinomycetes, as well as pathogenic fungi, which went hand-in-hand with a reduction in infectivity of plants and a reduction of pathogenic fungi and nematodes and hence less crop damage (Mitchell and Alexander, 1961; Oppenheim and Chet, 1992; Miller and Sands, 1977).

1. 10 Cloning of bacterial chitinase genes

1.10.1 Cloning and utilisation of *Serratia* chitinase genes

Serratia QMB 1460 was found to be the most active producer of chitinase from 100 bacterial isolates screened for activity by Monreal and Reece (1969). Roberts and Cabib (1982) purified chitinase from *Serratia marcescens* by affinity adsorption/desorbtion on chitin and characterised the chitinase activity by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as two major chitinases of molecular weights 58 and 52.5 kDa, and minor bands of 40.4 and 21.5 kDa. They further suggested that the enzyme was a good candidate for large scale degradation of chitin for industrial purposes. Horwitz *et al.* (1984) subsequently cloned endochitinase and chitobiase genes from a library of *S. marcescens* DNA cloned into the lambda vector, charon 4. A number of the plaques were positive both for chitinase and chitobiase and they concluded that these genes are probably linked in *S. marcescens*. The rationale for cloning the genes was to produce bacteria with higher chitin degrading potential, to offer an biological

alternative to inland dumping and hauling out to sea of waste chitinous materials.

In an effort to produce a novel approach for biocontrol, Fuchs *et al.* (1980) cloned the 57 kDa chitinase from *S. marcescens* using a cosmid library of genomic DNA. The idea was to clone the *S. marcescens* genes and introduce them into rhizoplane or phylloplane colonising bacteria. The chitinase gene was cloned on a 9.5kb genomic fragment, and the cosmid subsequently used to transform *Pseudomonas fluorescens* 701E1; significantly higher expression of chitinase was, however, observed in *E. coli* containing the construct than in *P. fluorescens*. Jones *et al.* (1986) reported that *S. marcescens* secretes something that can retard fungal growth and in order to investigate the role played in this antifungal activity by chitinases they cloned two chitinase genes from *S. marcescens* QMB1460. The chitinase genes were recovered from a cosmid library of *S. marcescens* DNA in an *E. coli* background on two separate plasmids, suggesting that they are not linked. They also showed no homology to each other as detected by Southern analysis. One clone expressed a 58 kDa chitinase and the other a 52 kDa chitinase that were named *chiA* and *chiB*, respectively. The *chiA* gene was sequenced after subcloning and an open reading frame of 1686bp was observed encoding a 61 kDa preprotein with a 3 kDa signal sequence. This gene was mutated by insertion of a neomycin phosphotransferase gene within the coding region and the insertion mutation was recombined into the parental *S. marcescens* strain. The resulting ChiA-mutant displayed reduced chitinase production (2 original genes encoding chitinase) and reduced inhibition of fungal spore germination, as well as reduced biological control of a fungal plant pathogen, *Fusarium oxysporum*, of pea plants. Further to this, the *chiA* gene was expressed in plants and inserted into *P. fluorescens* and *P. putida* yielding four strains of genetically manipulated *Pseudomonas* that had considerable chitinase activities (Jones *et al.*, 1986). Taylor *et al.* (1987) expressed the *chiA* gene in transgenic tobacco plants using a range of promoters; the resultant plants displayed increased resistance to the tobacco brown spot pathogen, *Alternaria longipes*. Lund and Dunsmuir (1992) subsequently compared secretion of the bacterial chitinase in transgenic tobacco plants with either the bacterial signal sequence or its replacement with a plant signal sequence and found better secretion with the latter. Jach *et al.*, (1992) also produced transgenic tobacco plants using the *S. marcescens* ChiA gene and detected its expression by PAGE. The bacterial chitinase was detected in leaves, stems and roots using ChiA specific antibodies and the transgenic seedlings were better protected against *Rhizoctonia solani* than control plants. Sundheim *et al.*, (1988) also cloned two chitinase genes from *S. marcescens* BJL200 and expressed them in

Pseudomonas. The deduced sequences were 95% and 96% identical to the *chiA* previously sequenced by Jones *et al.* (1986) and Koo *et al.* (unpublished), respectively (Brurberg *et al.*, 1994a). A one step purification scheme for the gene product has been described and use of the cloned gene for producing transgenic crop plants or transforming bacteria used in fermentation processes prone to fungal attack has been suggested (Brurberg *et al.*, 1994b). Shapira *et al.* (1989) also cloned a chitinase gene (*chiA*) from *S. marcescens* in *E. coli* and placed it under the control of a strong promoter. 90% of the enzyme was found to be secreted into the medium. The purified enzyme preparation caused rapid and extensive bursting of the hyphal tips of *Sclerotium rolfsi* and reduced incidence of disease in plants when added to the irrigation water. They suggested that the incorporation of the chitinase into a stabilised seed coating could protect the emerging seedling against soil borne pathogens, which may be of significant benefit since seedlings, rather than more mature plants, are most susceptible to damping off diseases. The plant symbiotic bacterium *Rhizobium meliloti* has also been transformed with the *chiA* gene of *S. marcescens* (Sitrit *et al.* 1993). Nodule extracts from plants infected with these transconjugants lysed the hyphal tips of the plant pathogen *Rhizoctonia solani,* whereas nodule extracts from plants infected with the wild type bacteria did not cause lysis. In addition to this, the plasmid was stable as judged by hybridisation analysis 45 days after inoculation and 99% of *Rhizobium* colonies carried the plasmid after growing the cells in liquid culture for 100 generations without antibiotics. It was therefore suggested that, as the result of their location inside the plant, symbiotic bacteria such as *Rhizobium spp.* which colonise the rhizosphere, as well as the root nodules may provide more efficient protection against plant pathogens than other rhizobacteria. Previous attempts to improve the biocontrol activity of rhizobacteria using cloned chitinase genes failed due to use of unstable constructs and subsequent loss of plasmids (Sundheim *et al.*, 1988), poor expression (Fuchs *et al.*, 1986) or inefficient gene expression.

The *chiB* chitinase gene has also been cloned and sequenced (Sundheim *et al.*, 1988; Harpster and Dunsmuir, 1989; Brurberg *et al.*, 1995), as well as the gene for N-acetyl-β glucosaminidase (Kless *et al.*, 1989), although the sequence for latter has not been reported. Chitinase B was shown to be exported to the periplasm without processing in *S.marcescens* BJL200, but was cytoplasmic in *E. coli* (Brurberg *et al.*, 1995) contradicting the earlier assumption of a 41 amino acid sequence signal peptide based on sequence analysis alone (Harpster and Dunmuir, 1989). The *chi B* of *S.marcescens* must therefore be exported by a secretion mechanism that differs from the general

secretion mechanism observed in prokaryotes.

Chitinase genes have also been isolated from *Serratia liquefaciens*. :- Two plasmids have been isolated from a DNA library constructed from the genome of this organism that conferred chitinase activity to *E. coli* (Joshi *et al.*, 1988). The 2 inserts were different in their physical maps and no hybridisation was found between them. One insert contained a chitinase gene *chi*A, the other contained a chitinase gene, *chi*B, a chitobiase gene *chi*C, and additional regulator genes, *chi*D and *chi*E.

1.10.2 Cloning of *Vibrio* chitinase genes.

Wortman *et al.* (1986) cloned the genes for endochitinase, chitobiase and possibly a related GlcNAc permease on a 5.1 kb chromosomal insert from *Vibrio vulnificus* in the vector pBR322, although they did not report the sequence; genes in this species of *Vibrio* were suggested to be organised in an operon. Similarly, chitinase, chitobiase and possibly permease activity have been reported for genomic inserts isolated from a clone library of hybrid plasmids containing *Vibrio harveyi* DNA (Soto-Gil and Zyskind, 1989; Horwitz *et al.*, 1984). However, the endochitinase activity was later shown to be due to cross reaction of lysozyme, which had been used to produce cell extracts, with the chitinase substrate. The chitobiase gene was subsequently sequenced (Soto Gil and Zyskind, 1989) and an open reading frame found encoding a 97.7 kDa chitobiase consisting of 883 amino acids. Perfect homology was found between six amino acids at the processing and modification region of the outer membrane lipoprotein of *E. coli* and amino acids 15-19 of the deduced pre-chitobiase sequence. Globomycin, a naturally occurring cyclic peptide and specific inhibitor of pro-lipoprotein signal peptide cleavage, prevented removal of the signal peptide from the N-terminus. This information suggests that the chitobiase is a lipoprotein and processed in a similar manner to the major outer membrane lipoprotein of *E. coli*. The sequence also displayed a high similarity score with human α-hexosaminidase, an enzyme implicated in Tay- Sachs disease (Soto-Gil and Zyskind, 1984). These authors also reported that the chitobiase gene was translocated to the outer membrane of *E. coli* and that since chitinase and chitobiase genes are not linked in *V. harveyi* they are therefore transcribed from different promoters and are unlikely to be co-ordinately controlled.

1.10.3 Cloning of *Aeromonas* chitinase genes.

Chen *et al.* (1991) cloned a chitinase gene from *A. hydrophila* JP101 in *E. coli* using the vector pBR322. Two chitinase positive clones were isolated out of 6,000 transformants grown on chitin agar plates, their plasmids containing inserts of 9.7 and 10.7kb. Subsequent analysis revealed that both plasmids contained an identical 4.5kb fragment coding for an 85 kDa chitinase. *E. coli* strains containing these contructs demonstrated similar repression of chitinase activity to the wild type strains. The chitinase was also secreted by *E. coli,* which contrasts with other *Aeromonas* proteins expressed in *E. coli,* such as amylase, aerolysin and a protease which accumulate in the periplasmic space. The authors suggest that the chitinase may be a useful model for fundamental studies on protein secretion, expression and regulation, in addition to applied research. An additional chitinase gene has been cloned from *Aeromonas caviae* (Sitrit *et al.*, 1995), a soil isolate known to secrete chitinases when growing on chitin as sole carbon source (Inbar and Chet, 1991). The gene was subcloned as a 4.5kb *Hin*dIII segment in the bluescript vector and a large open reading frame of 2595 base pairs, encoding a putative 865 amino acid exochitinase of 94kd was found. Comparison of the amino acid sequence with other chitinases revealed homology to chitinase A of *S. marcescens* and the chitinase of *Alteromonas* spp strain 07, as well as homology in regions of the C terminus to the last 40 amino acids of three cellulase gene products from *Bacillus* spp. These homologies suggest that the C terminal regions of *A. caviae* chitinase and *Bacillus* spp. cellulases are functionally related and may be involved in the ability of these enzymes to degrade highly hydrophobic substrates (Sitrit *et al.*, 1995). Ueda *et al.* (1992a, 1992b and 1994) isolated and purified chitinases I and II and β-N-acetylglucosaminidases from *Aeromonas* sp. No 10S-24, as well as cloning and sequencing the chitinase II gene. A cluster of chitinase genes has recently been cloned from another *Aeromonas* species on an 8kb fragment of cloned genomic DNA (Shiro *et al.*, 1996). Sequence analysis revealed four open reading frames, and the deduced amino acid sequences of open reading frames 4 and 3 coincided with the N-terminal sequences of chitinases I, VII and III respectively, that had previously been purified from *Aeromonas* sp. 10S-24 culture supernatant. The deduced amino acid sequences of ORF's 1-3 were similar to each other and consisted of domains displaying homology to chitin binding domains of *Bacillus circulans* chitinases A1 and D, domains displaying homology to the C-terminus of *Alteromonas* spp. 0-7 chitinase and chitinase II from *Aeromonas sp.* 10S-24, and catalytic domains separated by proline and / or threonine rich linkers (see figure 1.5 for open reading frames 1 and 3)).

1.10.4 Cloning of chitinase genes from *Alteromonas*.

The 07 strain of the marine organism *Alteromonas* secretes chitinase into the growth medium in the presense of chitin (Tsujibo *et al.*, 1993a). The chitinase gene was isolated from a genomic library of *Alteromonas spp.* DNA prepared in pUC18 as a 5kb insert; subcloning detected a 2.3kb *Sph*I/*Hin*dIII fragment that was necessary for chitinase activity, but this clone had a truncated C-terminus. A larger subclone was therefore sequenced containing the whole gene. *E. coli* containing this plasmid produced two chitinases of sizes 78 and 85 kDa. The N terminal sequences of these two chitinases were identical, suggesting that they arose from the same gene. Sequence analysis revealed a 2462bp open reading frame encoding a protein of 820 amino acids with a predicted molecular weight of 87kDa and a 21 amino acid signal sequence. The sequence was compared with other chitinase genes and showed 33.4% homology with *S. marcescens chi*A and 15.3% homology with *S. marcescens chi*B, although high sequence homology was not found in the N- or C- termini. The central portion of the *Alteromonas* chitinase also displayed high homology to chitinases from other bacterial and fungal origin as well as glycosidases from *Streptomyces plicatus* and *Flavobacterium spp.* The same authors suggested that the *Alteromonas* chitinase is divided into three domains, namely the signal sequence, a catalytic domain and the non-catalytic C-terminus.

1.10.5 Chitinases cloned from *Streptomyces.*

Streptomyces species are typical soil inhabitants which produce various carbohydrolases, including chitinase, expression of which is repressed by glucose and induced by chitin. Miyashita *et al.* (1991) isolated strains of *Streptomyces* with multiple chitinases and high chitin degrading activities from soil. Genetic control was analysed by self cloning three chitinase genes from *Streptomyces lividans* by screening for increased chitinase activity. Four chitinases were identified with molecular masses of 36, 46, 65 and 41 kDa, named chitinases A-D, respectively. Chitinases A and B were produced by individual clones whereas chitinases C and D were specified by a single clone; chitinases C and D were also similar in terms of their pH requirements for activity and their mode of substrate digestion. Chitinases A and B were further characterised as exochitinases, whereas chitinases C and D were endochitinases. The chitinase C gene was sequenced by Fujii and Miyashita (1993), revealing two open reading frames having opposite orientations. Northern analysis revealed that only one of these open reading frames was transcribed and was induced by chitin and repressed

by glucose. Furthermore, the N-terminal sequence of chitinases C and D were identical and therefore chitinase D is likely to be a proteolytic derivative of chitinase C. Chitinase C is encoded by a 1857bp open reading frame and has a deduced amino acid sequence of 619 amino acids.

A chitinase purified from the culture filtrate of *Streptomyces erythraeus* (Kamei *et al.*, 1989) was shown to have a molecular weight of 30 kDa and a pI of 3.7, with optimal activity at pH5. The mode of binding of this chitinase to its substrate was similar to that of hen eggwhite lysozyme and *Streptomyces erythraeus* lysozyme. The chitinase sequence, obtained by sequencing tryptic digests of the protein, showed no homology to any other reported chitinase except at the proposed active site of bacterial chitinases. Thus, *S. erythraeus* chitinase has a unique structure.

Robbins *et al.* (1988) cloned the chitinase genes of *Streptomyces plicatus* utilising the lamda cloning vector EMBL-4. Positive clones were shown to contain chromosomal DNA of 12-17kb. These fragments were subcloned in pUC18 and sequenced; unfortunately the sequences have not been published. The cloned DNA codes for a 63 kDa chitinase which was compared to three other *S. plicatus* chitinases by activity; chitinases of 47 and 61 kDa were exochitinases and those of 49 and 63 kDa were endochitinases.

Berger and Reynolds (1958) characterised the chitinase system of *Streptomyces griseus* as being composed of two chitinases and a chitobiase. The chitinases had the same substrate specificities suggesting that one may be the proteolytic degradation product of the other; or alternatively they may be the products of separate genes (Berger and Reynolds, 1958).

In the course of a screening programme, *Streptomyces olivaceoviridis* was identified as a highly efficient degrader of crystalline chitin (Beyer and Diekmann, 1985) and five chitinases (20.5, 30, 47, 70 and 92 kDa) have been purified to homogeneity from this organism (Romageura *et al.*, 1992). Blaak *et al.* (1993) also cloned an exochitinase gene from this bacterium that encoded a chitinase that produced only chitobiose from chitin and chitin oligosaccharides. Hydrophobic cluster analysis of the deduced amino acid sequence of the protein encoded by this gene with other chitinolytic enzymes showed that this chitinase has the highest overall amino acid identity with *Bacillus circulans* chitinase D, enabling prediction of the domain structure of the enzyme.

A thermostable chitinase has been purified from *Streptomyces thermoviolaceus* OPC-520. The enzyme displayed a high optimum temperature (70-80^{0}C) as well as a high

optimum pH (8-10) Tsujibo *et al.* (1993b). The gene encoding this chitinase was subsequently cloned and sequence analysis revealed a high sequence homology (74%) to *chi* 63 from *S.plicatus*

1.10.6 *Bacillus* chitinases

Watanabe *et al.* (1990a) described the chitinase system of *Bacillus circulans* WL-12 as being composed of a number of chitinases of molecular weights 74, 69, 52, 39, 38, and 38kDa; named A1, A2, B1, B2, C and D from the largest, to the smallest respectively. Chitinases A1 and A2 had identical N-terminal amino acid sequences, as did B1 and B2, indicating that each pair of chitinases was the product of a single gene; the remaining two enzymes have different N-terminal sequences. Chitinase A1 was shown to be capable of binding to chitin, but this property was lost when A1 was proteolytically cleaved to chitinase A2. Chitinase A1 was described as the key enzyme in the degradation of chitin and was classified as an exochitinase

The gene encoding chitinase A1 has been cloned into *E. coli* HB101 in the plasmid vector pKK223-3 and sequence analysis revealed the presence of a 2097 bp open reading frame coding for the precursor of A1 on a 4kb fragment of chromosomal DNA (Watanabe *et al.*, 1990a). This precursor contained a long signal sequence of 41 amino acids with an extremely long 15 amino acid hydrophilic N-terminus. The N-terminal two thirds of the deduced amino acid sequence of chitinase A1 gave a 33% match to chitinase A of *S. marcescens*.

The gene coding for chitinase D was found to be located immediately upstream of the *chi*A gene, encoding chitinase A1 (Watanabe *et al.*, 1992), and the two enzymes are similar in that they both display high affinities for colloidal chitin. Sequence analysis of chitinase D revealed an open reading frame encoding a protein of 488 amino acids. The end of the *chi*D sequence is located 103 bp upstream from the start of the *chi*A gene and the genes show high homology between the N-terminal one third of chitinase D and the C-terminal one third of chitinase A1. A central 73 amino acid portion of chitinase D also displayed considerable sequence homology to other bacterial chitinases, class III higher plant chitinases and *Streptomyces* endo-β- *N*-acetylglucosaminidases, as well as to the *Kluyveromyces lactis* killer toxin α subunit (Watanabe *et al.*, 1992).

1.10.7 Cloning of other chitinase genes

Chitinase genes have been cloned from *Janthinobacterium lividum* (formerly *Chromobacterium lividum*) (Gleave *et al.*, 1995), *Cellvibrio mixtus* (Wynne and Pemberton, 1986), *Clostridium thermocellum* (Accession number Z68924) and *Kurthia zopfii* (Accession number D63702) although, as yet, the latter two have not been published.

The chitinase of *Janthinobacterium lividum* is 665 amino acids long, including a 24 amino acid signal sequence, producing a 69kDa mature chitinase. Its structure consists of two putative chitin binding domains showing homology to similar domains in chitinases A1 and D of *B.circulans*. The catalytic domain is separated from these chitin binding domains by linker regions of serine-threonine repeats. The chitinase gene from *C. mixtus* was cloned as part of a gene cluster coding for cellulase, chitinase, amylase and pectinase; the sequence of the chitinase gene has not yet been published. During the writing of this thesis, other bacterial chitinase genes have been cloned and sequenced including; the gene encoding *Clostridium paraputrificum* chitinase ChiB (Morimoto *et al.*, 1997), two genes encoding 54 kDa and a 22 kDa chitinases of *S. marcescens* (Gal *et al.*, 1997) and a gene encoding chitinase ChiA from *Enterobacter agglomerans* (Chernin *et al.*, 1997). Two chitinases produced by *Pseudomonas aeruginosa* K-187 have also been characterised. The enzymes displayed antibacterial and cell lysis activities with many kinds of bacteria in addition to chitinase activity, this being the first report of a bifunctional chitinase/lysozyme from a prokaryote (Wang and Chang, 1997).

1.11 Control of chitinase expression

There is, therefore, a very large amount of information regarding the cloning and sequencing of chitinase genes from a wide variety of bacteria. However, although microbial chitinases are typically induced by chitin and repressed by glucose, there is actually very little information available in the literature on the control of expression of such genes. The *Serratia marcescens* chitinase A gene has a catabolite activating protein binding site in the vicinity of the promoter, indicating control of expression by catabolite repression (Jones *et al.*, 1986). *Streptomycetes* chitinases are also under catabolite control; however, the mechanism of repression in *Streptomyces* seems to be quite different from the c-AMP dependant system of *E. coli*. Sequence analysis of *Streptomyces lividans* chitinase C gene revealed two identical 12bp direct repeat sequences in the promoter region of c*hi*C overlapping the putative -35 sequence (Fujii

and Miyashita, 1993). Similar direct repeats found in the promoter regions of chitinase genes *chi*63 and *chi*35 of *S. plicatus* (Delic *et al.*, 1992) and the chiA of *S. lividans* (Fujii and Miyashita, 1993) are also suggested to be involved in the regulation of the expression of these genes. Very recently the function of the 12bp direct repeats have been studied in *Streptomyces* chitinase-63 (Ni and Westpheling, 1997). Some base changes within these direct repeat sequences resulted in constitutive expression of chitinase, suggesting that this sequence is an operator for negative regulation. Other base changes resulted in loss of glucose repression while retaining the requirement for chitin induction. Together these observations suggest that the direct repeat sequence facilitates both chitin induction and glucose repression. The chitin utilisation regulon of *Serratia liquefaciens* is that with the best characterised control system to date (Joshi *et al.*, 1988). Transposon mutagenesis and deletion analysis of an 8kb fragment of genomic DNA known to contain chitinase B and chitobiase C genes, identified a region *chi*D, the absence of which led to higher expression of chitinases A and B in this bacterium, indicating that the region may code for a repressor. Loss of function of another adjacent region, *chi*E, prevented induction unless a *chi*E$^+$ strain was grown in close proximity, suggesting that the deleted gene may code for a protein that is involved in the synthesis of the inducer. Another repressor (Reg1) has recently been identified in *Streptomyces lividans* (Nguyen *et al.*, 1997). Chromosomal disruption of *reg1* increased the expression of several genes including chitinase.

1.12 Structure / function of chitinase genes and chitinases.

The number of chitinase genes that have been cloned and sequenced in general, and bacterial chitinase genes in particular, has increased massively during the last 5-6 years. This growth has led to a concomitant increase in the information regarding the structure and function of the products that the genes encode, as well as the organisation of the genes themselves. X-ray structures are now available for two plant chitinases (Hart *et al.*, 1993; Terwisscha van Scheltinga *et al.*, 1994), as well as one bacterial chitinase (Perrakis *et al.*, 1994) and the mechanisms of action, as well as the groups involved in catalysis, are steadily being revealed. This section describes the various domains found in chitinases and their proposed function and organisation.

1.12.1 Fibronectin type III domains

Most of the work concerning the organisation and function of putative peptide domains in bacterial chitinases has focused on *Bacillus spp.*, *Streptomyces spp.* and *S. marcescens* chitinases. The cloning and sequencing of chitinase A1 from *Bacillus circulans* WL-12, revealed repeating 95 amino acid units (R1 and R2) in the C-terminus that are 70% identical to each other (Watanabe *et al.*, 1990a). These repeats were found to have statistically significant homologies to so called type III homology units located in the middle sections of bovine and human fibronectins (see figure 1.3 for an alignment of various fibronectin type III domains). Furthermore, in an alignment of 16 type III homology units of fibronectins, the amino acid residues that were identical in 8 or more of these units were also found in the tandem repeats of chitinase A1 (Kornblihtt *et al.*, 1985). Fibronectin is a multifunctional, extracellular matrix and plasma protein that plays a central role in cell adhesion. The middle part of fibronectin contains a DNA binding domain, cell attatchment site, and the main heparin binding site (Ruoslahti, 1988). The observation of these domains in this bacterial chitinase was the first finding of prokaryotic type III repeating units. A similar individual fibronectin type III domain was subsequently found in chitinase D in the same strain of *B. circulans* (Watanabe *et al.*, 1992). This time the domain was found at the N terminal end of the putative amino acid sequence from positions 55-150, and may have arisen by sequence duplication. In experiments to determine the roles of the C-terminal domain and type III domains of chitinase A1 from *B. circulans*, using sequential deletions of the C-terminal segments of the chitinase gene, Watanabe *et al.* (1994) found that the fibronectin type III domains were not required for chitin binding activity of the enzyme, but were required for efficient hydrolysis of crystalline chitin. Colloidal chitin hydrolysing activity was decreased stepwise with deletions of fibronectin type III domains. Modified chitinases lacking one type III module but containing the C-terminal domain displayed significantly decreased hydrolysing activity; deletion of both domains reduced hydrolysing activity to that of chitinases lacking the C-terminal domain. Fibronectin type III domains have since been found in a number of other chitinases, namely *Streptomyces lividans* chitinases A, B and C, S*treptomyces plicatus* chitinase 63 (Watanabe *et al.*, 1993) and S*treptomyces olivaceoviridus* chitinase (Blaak *et al.*, 1993). Similarly, fibronectin type III domains have been found in various bacterial carbohydrases including poly-β-hydroxybutyrate-depolymerase from *Alcaligenes faecalis* (Saito *et al.*, 1989), CenB cellulase from C*ellulomonas fimi* (Meinke *et al.*, 1991), a cellulase from *Cellulomonas flavigena*, an exo-poly-α D-galacturonosidase

from *Erwinia chrysanthemi*, a bifunctional α-amylase-pullanase from *Clostridium thermohydrosulfuricum*, and a maltopentose-producing amylase from an alkalophilic Gram-positive bacterium (Blaak *et al.*, 1993). In addition, the crystal structure of *S. marcescens* ChiA has recently been solved by Perrakis *et al.* (1994). The N-terminal domain displayed structural similarity to the fibronectin type III domain, even though the sequence has no homology. The sequence of the N-terminus also displayed homology to the N-termini of chitinases from *Aeromonas caviae* and *Alteromonas* species 07 indicating similar structures. The presense of these domains in this number of carbohydrases suggests that they are important in their interaction with their respective substrates.

Figure 1.3 Sequence alignment of fibronectin type III domains (adapted from Blaak *et al.*, (1993))

Alignment of the consensus amino acid sequence of type III homology units of fibronectin (Kornblihtt *et al.*, 1985) with corresponding characteristic amino acids from :- (1) exochitinase exo-Chi 01 of *S. olivaceoviridus* (Blaak *et al.*, 1993), (2) chitinase D of *B. circulans* (Watanabe *et al.*, 1992), (3) and (4) chitinase A1 of *B.circulans* (Watanabe *et al.*, 1990a) (R1 and R2 indicate repeats 1 and 2 respectively), (5) chitinase 63 of *S.plicatus* (Robbins *et al.*, 1992), (6) a poly(3-hydroxybutyrate) depolymerase of *A. faecalis* (Saito *et al.*, 1989) and (7-9) three repeats of the cellulase CenB of *C. fimi* (Meinke *et al.*, 1991).

Residues identical in 50% or more of the sequences are highlighted and displayed in the consensus.

Figure 1.3 sequence alignment of fibronectin type III domains

		POSITION	
	type III consensus		P X X P X X L X X T - X X T X X S X X - - - - - L S W X A S T D X X -
1	S.oliva exo Chi 01	170	P A P P T G L R - T G S V T A T S V A - - - - - L S W S P V T G A - -
2	B.circulans ChiD	109	P T V P A G L T S S - L V T D T S V N - - - - - L T W N A S T D N V -
3	B.circulans ChiA1(R1)	545	P S V P G N A R S T - G V T A N S V T - - - - - L A W N A S T D N V -
4	B.circulans ChiA1(R2)	640	P T A P T N L A S T - A Q T T S S I T - - - - - L S W T A S T D N V -
5	S.plicatus Cht 63	196	P G D E A P S A P G - T P T A S N I T D T S V K L S W S A A T D D K
6	A.faecalis Phbuty	362	L S W N A V A N A S -
7	C.fimi CenB (Fn3a)	669	L S W A A S T D A G S
8	C.fimi CenB (Fn3b)	766	L A W A P S T G D P A
9	C.fimi CenB (Fn3c)	865	L T W T A S T D S G G

	type III consensus		- - - T G Y X V Y R X X X X X X X X X X - - T T A X X X X X X X X T X
1	S.oliva exo Chi 01	197	- - - T G Y A V Y R D G V K V A T A S G - - T S A - - - - - - - - - T V
2	B.circulans ChiD	137	- G V T G Y E V Y R N G T L V A N T S T - - T T A - - - - - - - - - V V
3	B.circulans ChiA1(R1)	573	- G V T G Y N V Y N G A N L A T S V T G - - T T A - - - - - - - - - T I
4	B.circulans ChiA1(R2)	668	- G V T G Y D V Y N G T A L A T T V T G - - T T A - - - - - - - - - T I
5	S.plicatus Cht 63	229	- G V K N Y D V L R D G A T V A T V T G - - T T Y - - - - - - - - - T D
6	A.faecalis Phbutyr	372	- - - - - S Y G V Y R N G S K V G S A T A - - T A Y - - - - - - - - - T D
7	C.fimi CenB (Fn3a)	679	- G V A G Y G L Y R V Q G T T Q T L V G T T T A A A - - - - - - - - - Y I
8	C.fimi CenB (Fn3b)	776	- - V S G Y D V L R V Q G T T T T V V A Q T T V P T - - - - - - - - - V T
9	C.fimi CenB (Fn3c)	875	S G L A G Y E V L R V S G T T Q T L V A S P T T A A - - - - - - - - - T V

	type III consensus		X - G L T X G T X Y X Y X V K A X D - X A G N - - - S A A S X X V X X T T
1	S.oliva exo Chi 01	219	T - G L T P D T A Y A F Q V A A V N - G A G E - - - S A K S A T V T A T T
2	B.circulans ChiD	161	T - G L T S G T T Y V F T V K A K D - A A G N L - - S A A S T S L S V T T
3	B.circulans ChiA1(R1)	597	S - G L T A G T S Y T F T I K A K D - A A G N L - - S A A S N A V T V S T
4	B.circulans ChiA1(R2)	692	S - G L A A D T S Y T F T V K A F D - A A G N V - - S A A S N A V S V K T
5	S.plicatus Cht 63	253	N - G L T K G T D Y S Y S V K A R D T G D Q T G - - P A S G - S V K V T T
6	A.faecalis Phbutyr	393	S - G L I A G T T Y S Y T V A V D P T A G E S Q P S A A - - - V S A T T
7	C.fimi CenB (Fn3a)	707	L R D L T P G T A Y S Y V V K A K D - V A G N V - - S A A S A A V T F T T
8	C.fimi CenB (Fn3b)	803	L S G L T P S T A Y T Y A V R A K N - V A G D V - - S A L S A P V T F T T
9	C.fimi CenB (Fn3c)	904	A L G L T P A T A Y S Y V V R A K D - G A G N V - - S A V S S P V T F T T

1.12.2 Putative chitin binding domains within chitinases.

The work of Watanabe *et al.* (1994) regarding the roles of the C terminal domain and fibronectin type III domains of *Bacillus* chitinase A1, has also shed light on the chitin binding ability of this enzyme. Chitin binding assays of modified chitinases to regenerated chitin found that enzymes containing the C-terminal domain were able to bind to chitin, whereas those lacking the C-terminal markedly lost this activity. It was concluded that the C-terminal domain plays a major role in the chitin-binding activity of the enzyme, but Watanabe and his fellow workers (1994) also suggested that the catalytic domain itself has affinity for chitin, since chitinases lacking the C-terminal domain also possess some ability to adsorb to regenerated chitin when compared with a control protein (bovine serum albumin).

The chitin binding activity of *Bacillus* chitinase A1 also appears to be important for complete digestion of insoluble chitin. Chitinases lacking fibronectin type III domains but containing the C-terminal domain formed transparent clearing zones with well defined edges on agar plates containing colloidal chitin, when visualized by staining. In contrast, the zones formed by chitinases lacking the C-terminal domain were faint and fuzzy. Chitinase D of *B. circulans* has a N-terminal domain that shares 61.7% identity to the C-terminal domain of chitinase A1 and this has also been shown to be essential for chitin binding activity (Watanabe *et al.*, 1994). Alignment of the putative chitin binding domains of chitinase A1 and D with a similar section of a *Aeromonas sp* chitinase and the tandem chitin binding domains of *Janthinobacterium lividum* displays a number of common residues which may be involved in binding of the enzyme to chitin (figure 1.4 A). The sequence similarity between the putative chitin binding domain of *S. marcescens* ChiA and the N-terminal domain of chitinase 85 of an *Alteromonas sp.* is also shown (figure 1.4 B), as is the similarity between the N-terminus of *S.lividans* chitinase C and cellulose-binding domains of exoglucanase from *Cellulomonas fimi* (figure 1.4 C). These observations suggest that chitin binding domains may be a general feature of many bacterial chitinases. Similar substrate binding domains have been found in a number of other carbohydrases including cellulose binding domains in exoglucanase Cex and endoglucanase CenA of *C. fimi* as well as the cellobiohydrolases CBH1 and CBH11 of *Trichoderma reesi (*Gilbert *et al.*, 1990; Meinke *et al.*, 1991*)*. Substrate binding domains have also been found in β-1,3 glucanase of *B. circulans* WL-12 and chitin binding domains have also been found in yeast and plant chitinases; however, these domains do not seem to play a role in the hydrolysing efficiency of the enzyme. In the case of the yeast chitinase, removal of the

binding domain increased the rate of chitin hydrolysis, whereas the loss of the chitin binding domain of tobacco class 1 chitinase failed to affect the activity of the enzyme, although it did reduce its anti-fungal activity to one third. Therefore, the chitin binding domains are not always important for maximum chitinase activity, but most likely confer unique biological properties to the particular enzymes in different organisms.

Figure 1.4 Alignment of possible chitin-binding domains found in several bacterial chitinases

(A) Alignment of the chitin binding domain of (1) *B.circulans* chitinase A1 (C-terminal domain), with (2) *B.circulans* chitinase D chitin-binding domain (N-terminal domain), (3) the C-terminal region of chitinase II from an *Aeromonas sp.* and (4) and (5) the two putative chitin binding domains of *Janthinobacterium lividum.*
(B) Alignment of (1) the N-terminal region of chitinase A from *S. marcescens* with the N-terminal region of chitinase-85 from an *Alteromonas spp.*
(C) Alignment of (1) section of chitinase C from *S.lividans* and (2) the cellulose-binding domain of exoglucanase (Cex) from *C.fimi*
Residues conserved in four of five (A), or two of two (B) and (C) aligned sequences are blocked and highlighted.

Figure 1.4 Alignment of possible chitin binding domains found in several bacterial chitinases

(A)

```
                                POSITION
1.B.circulans A1 CT    652   G V S A W Q V N T A Y T A G Q L V T Y N G K T Y K C L Q P H T S L A G - - W E P S N V P A L W Q L Q
2.B.circulans D  NT     31     A A Q W Q A G T A Y K Q G D L V T Y L N K D Y E C I Q P H T A L T G - - W E P S N V P A L W K Y V
3.Aeromonas  sp. CT    493   G C A A W A E G N T Y T A G T C A S Y G G K D Y V A Q V T H T A Y V G A N W N P A A T P T L W K L K
4.J.lividum  Chi69a     55   A C V P W Q E G G T Y N A G T V V T Y L G G N Y T A L V T Q T D H V G S G W N P V S T P S L W S A G
5.J.lividum  Chi69b    154   C A L A W A A G T A Y S A G A T V S Y A G T N Y R A - - - - - - - - - -N Y W T Q G D N P S T S S G G
```

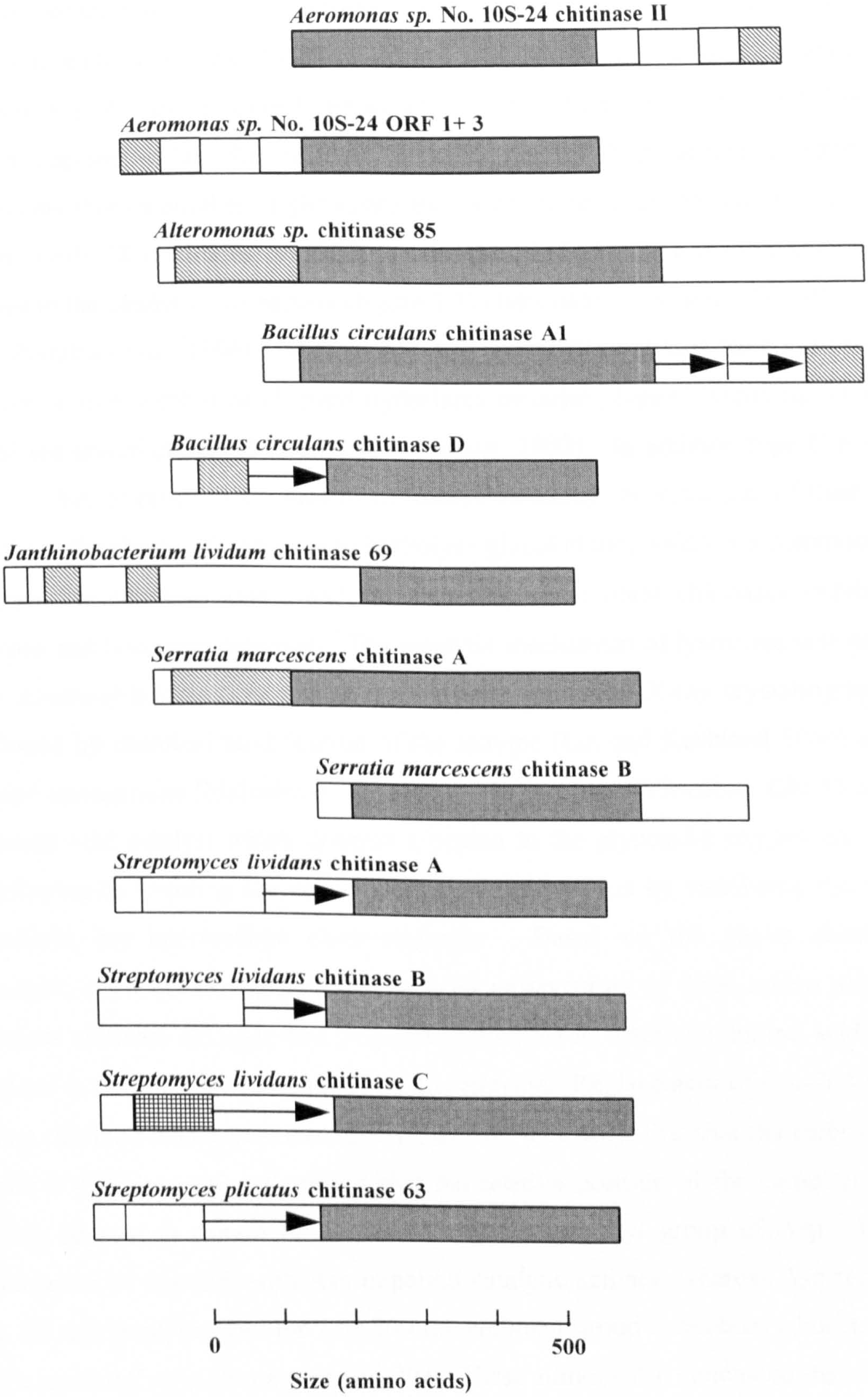
(B)

```
                                POSITION
1.S.marcesans ChiA NT   25   A A P G K P T I A W G N T K F A I V E V D - Q A A T A Y N N L V K V K N A A D V S V S W N L W N G D A G
2.Alteromonas      NT   22     A P S T P T L D W Q P Q Q Y S F V E V N V D G L G S Y K Q L V K A K D V V D I S I K W N A W S G S G G

                             77   T G P K I L L N G K E A W S G - - P S T G S S G T A N F K V N K G G R Y Q M Q V A L C N A D G C T A S D
                             74   D N Y K V Y F D D L L V N Q G S L P A G T K S G V V Q F P Y T K S G R H Q L Y L E L C E G T V C A R S A

                            129   A T E I V V A D T D G S H L P P L K E P L L E K N K P Y K Q
                            126   G K E I V I A D T D G A H L A P L P M N V D P N N R N N G T
```

(C)

```
                                POSITION
1.S.lividans NT         31   A T S A T A T F A K T S D W G T G F G G S W T V K N T G T T S L S S W T V E W D F P T G T K V T S A W D
2.C.fimi     Cex       380   A - G C Q V L W G V - N Q W N T G F T A N V T V K N T S S A P V D G W T L T F S F P S G Q Q V T Q A W S

                           83   A T V T N S G D H W T A K N V G W N G T L A P G A S V S F G F N G S G P G S - - - P S N C K L N G G S C
                          432   S T V T Q S G S A V T V R N A P W N G S I P A G G T A Q F G F N G S H T G T N A A P T A F S L N G T P C
```

Figure 1.7 Domain structure of various chitinases



1.12.4 Catalytic domains

Chitinases have been grouped into two families using sequence similarities (families 18 and 19 of the glycosyl hydrolases). All bacterial chitinases belong to family 18 and hence probably have a similar mode of catalysis (Henrissat, 1990, 1991 and 1993). The catalytic domains of bacterial chitinases, class III plant chitinases, fungal and yeast chitinases, endo-β-N-acetylglucosaminidases and some viral chitinases share weak sequence homologies in general (Blaak *et al.*, 1993; Perrakis *et al.*, 1993), although specific regions within the catalytic domains display high sequence homologies. Alignments show a number of glutamate and aspartate residues that are well conserved among family 18 chitinases providing a strong indication of the involvement of these residues in the catalytic mechanism (figure 1.5) (Henrissat, 1990 and 1991; Blaak *et al.*, 1993; Perrakis *et al.*, 1994). Interestingly Asp and/or Glu residues have been found to be catalytic in a number of glycosyl hydrolases including type-C lysozyme which also hydrolyses glycol chitin (Phillips, 1967; Sinnott, 1990). In addition type C lysozyme has a number of other similarities to chitinases including the structures of their natural substrates, the ability of lysozyme to hydrolyse glycol chitin, which is a commonly used substrate for chitinase assay, and the fact that some plant chitinases exhibit both chitinase and lysozyme activities. The catalytic mechanism of lysozyme was proposed from structural information gained from atomic resolution X-ray crystallography and confirmed by chemical modification of the enzyme (Lin and Koshland 1969) and site-directed mutagenesis (Malcolm *et al.*, 1989). The mechanism involves Glu-35 acting as a general acid catalyst which donates a proton to the glycosidic oxygen and Asp-52 contributing to lowering the energy barrier of the reaction by stabilising the transient carbonium ion intermediate electrostatically. Based on the above observations, Watanabe *et al.* (1993) utilised site directed mutagenesis of three amino acids in *B. circulans* chitinase A1 (Ser-160, Asp-200, Glu-204) to determine amino acid residues involved in the catalytic mechanism of this enzyme. Replacement of Glu-204 with Gln or Asp rendered the enzyme essentially inactive, indicating first that the carboxyl group of Glu-204 is essential and second that the relative position of the carboxyl group is critical, because it cannot be substituted by the carboxyl group of Asp. However, replacement of Asp-200 with Asn impaired catalytic activity, whereas Asp replaced by Glu did not, implying that for Asp-200 the carboxyl group is necessary but its position is not as critical as in the case of Glu-204. These authors also concluded that the role of the Ser-160 residue was unclear since a mutant enzyme with Ser-160 replaced by Ala retained 10% activity, indicating that the hydroxyl group of Ser-160 is not absolutely

required for activity. These results therefore indicated that Glu-204 and Asp-200 are directly involved in the catalytic mechanism of chitinase A1; however, Perrakis *et al.* (1994) later questioned the role of Asp-200. The results are, however, consistent with chitinase functioning in a similar manner to lysozyme. More recently the crystal structures have been reported for two plant enzymes, one belonging to family 19 of the glycosyl hydrolases (Hart *et al.*, 1993) the other belonging to family 18 (Terwisscha van Scheltinga *et al.*, 1994). The crystal structure has also been determined for chitinase A from *S. marcescens* (See figure 1.6 for ribbon diagrams) showing it to be comprised of three domains. The amino terminal domain consists only of β-strands, displaying structural similarity to fibronectin type III folds. This domain connects, through a hinge region, to the largest domain which is a catalytic α/β barrel; the third domain has an α+β fold and is formed by an insertion in the α/β barrel. The α/β barrel contains a long groove, at the C-terminal ends of the β-strands, that forms the substrate binding site, consistent with all known enzymes containing an α/β barrel. The active site was identified by solving the structure of the enzyme in the presence of an oligomer of its natural substrate. Unfortunately, the poor quality of the complex did not permit any insight into the mode of substrate binding nor identification of the structural features determining the specificity for the chitin polysaccharide. The catalytic residues were found to include a Glu at position 315 and possibly an Asp at position 391, the former being consistent with the results of the site-directed mutagenesis of *B. circulans* chitinase A1 (Watanabe *et al.*, 1993) although Perrakis *et al.* (1994) reported that mutation of Asp-200 in this enzyme to Asn could not be shown to be directly associated with the reaction mechanism. The equivalent residue of *S. marcescens* Asp-391 was not mutated in *B. circulans* chitinase (Watanabe *et al.*, 1993) and therefore cannot be deemed to be catalytic with absolute confidence although structural data indicate that Asp-391 is the only charged residue, other than Glu-315, in the region where the sugar ring is bound. Perrakis *et al.* (1994) concluded that Glu-315 would be protonated at acidic pH and would be the proton donor in the general acid-base catalytic mechanism; Asp-391 would be in a more hydrophilic environment and is predicted to have a negative charge at acidic pH, which would allow it to stabilise the carboxy-anion intermediate. The stereochemistry is consistent with a catalytic mechanism similar to that of lysozyme, i.e. general acid-base catalysis.

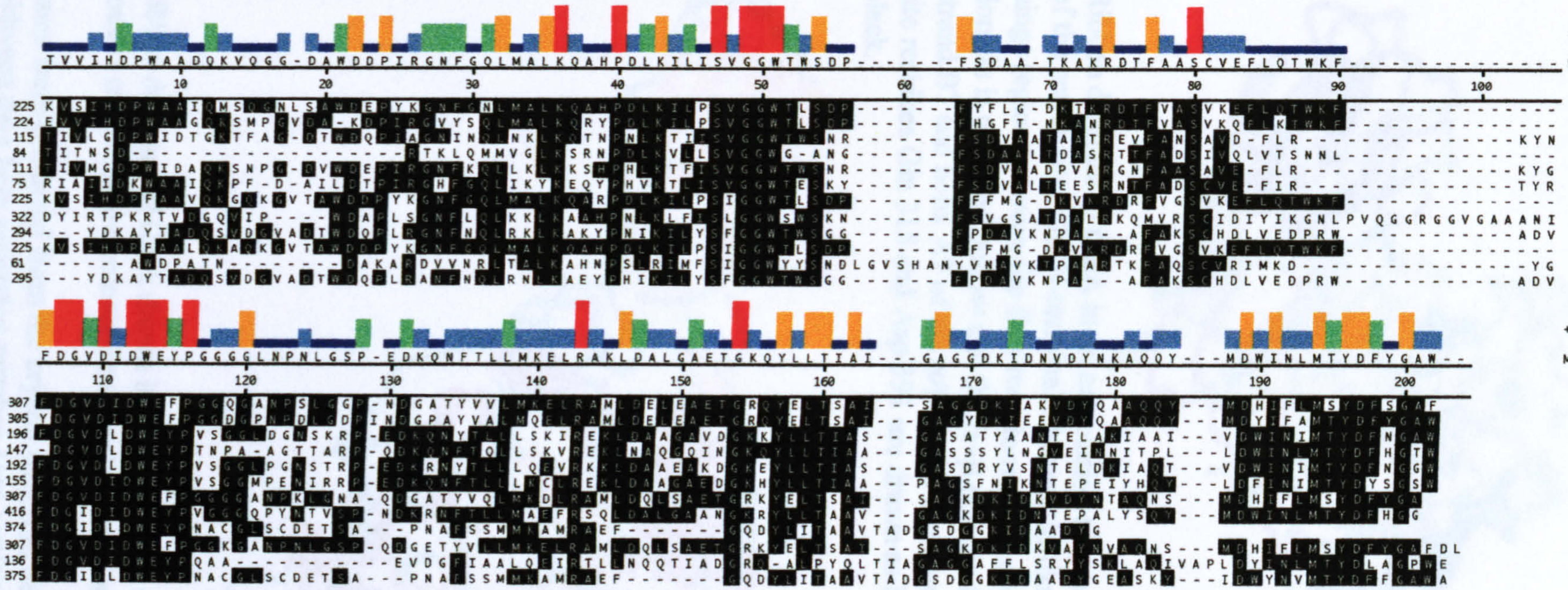
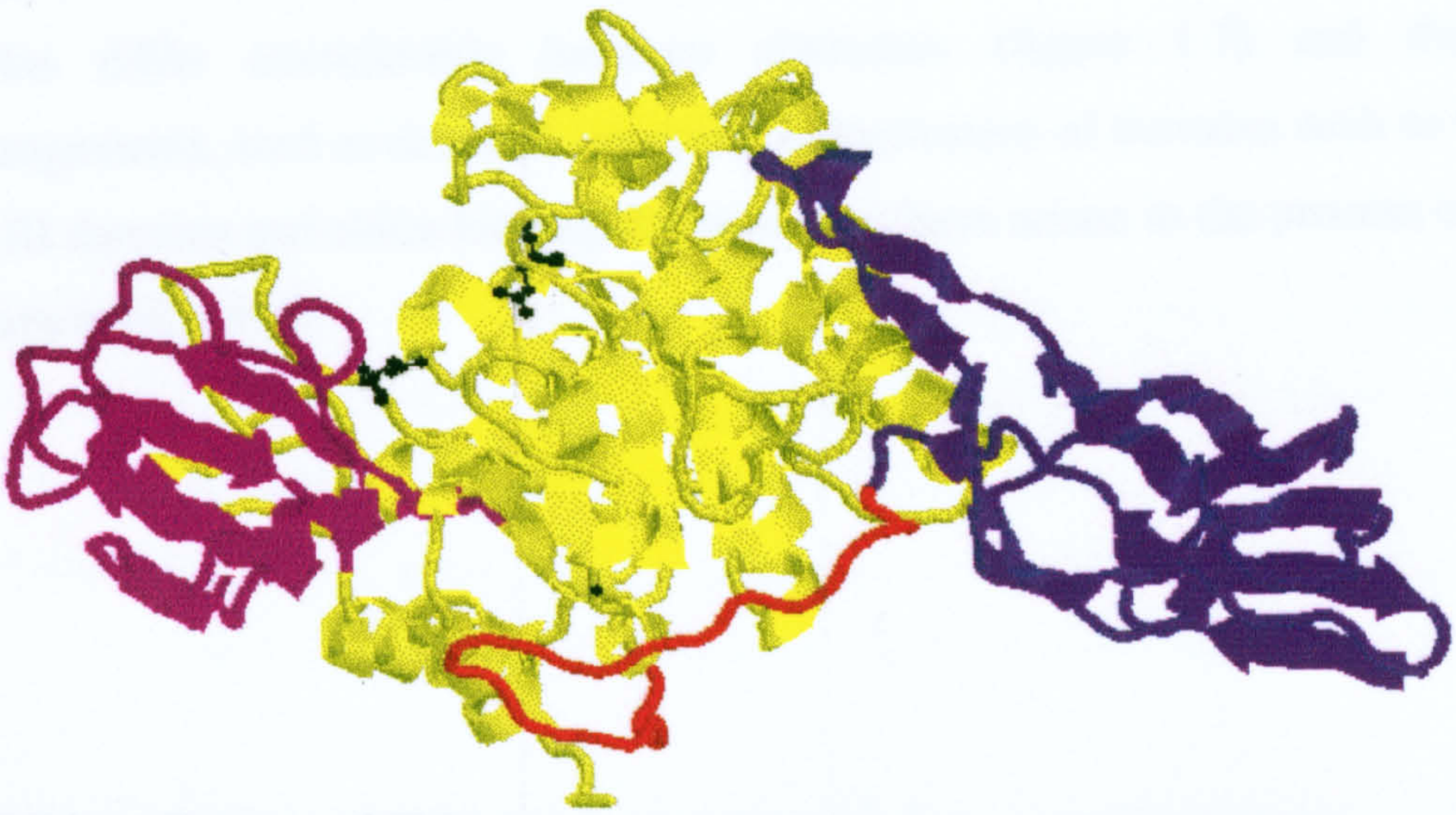


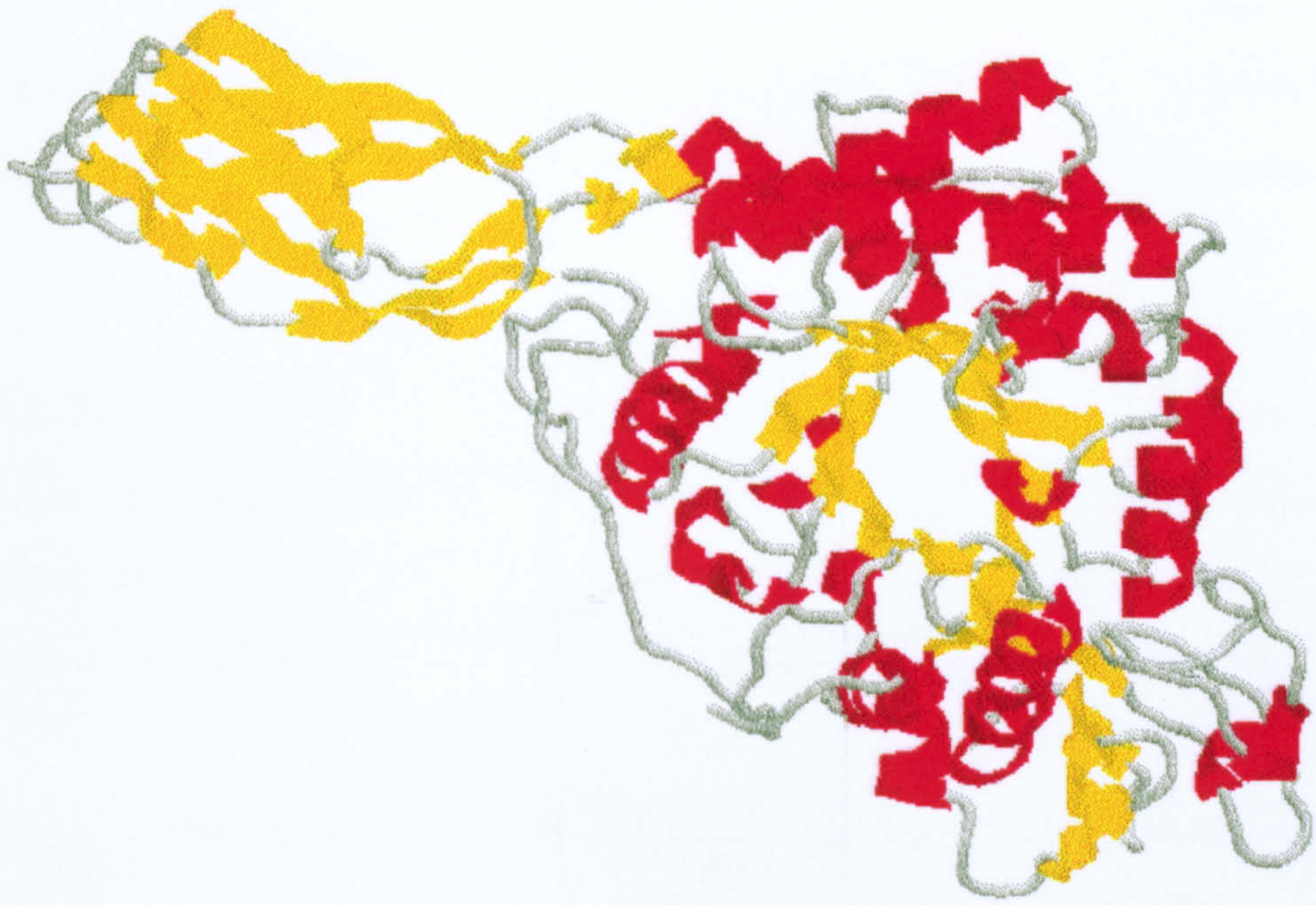
Figure 1.7 Alignment of section of catalytic domain of family 18 bacterial chitinases

The catalytic domains of 12 bacterial chitinases; 1. *Aeromonas caviae* ChiA (Sitrit *et al*, 1995; 2. *Alteromonas* Chi85 (Tsujibo *et al*, 1993); 3. *Bacillus circulans* ChiA (Watanabe *et al*, 1990); 4. *Bacillus circulans* ChiC (Alam *et al*, 1995); 5. *Bacillus licheniformis* Chi59 (Takayanagi *et al*, 1991); 6. *Clostridium thermocellum* Chi (Acc N0 Z68924); 7. *Enterobacter agglomerans* ChiA (Chernin *et al*, 1997); 8. *Janthomonas lividum* Chi69 (Gleave *et al*, 1995); 9. *Streptomyces lividans* Chi66 (Miyashita and fuji 1993); 10. *Serratia marcescens* ChiA (Jones *et al*, 1994; 11. *Serratia marcescens* ChiB (Sundheim *et al*, 1988); 12. *Streptomyces plicatus* Chi63 (Robbins *et al*, 1992), were aligned with the clustal V algorithm (Higgins and Sharp, 1989) using Lasergene software. The consensus sequence (majority) shows the most frequent amino acid at each position. Amino acids that are identical to the consensus are shaded black. The strength of the conservation at a given position is indicated by bars of increasing height and colour blue- red above the consensus sequence

Figure 1.6 Ribbon diagrams of *Serratia marcescens* ChiA



(A) Ribbon diagram of ChiA in an orientation to show the three domains and the groove of the active site; The N-terminal fibronectin-like domain is coloured blue, the hinge region between the first two domains is coloured red; the catalytic α/β barrel domain is coloured yellow and the α+β domain formed by an insertion between strand B7 and helix A7 of the α/β barrel is coloured purple. The proposeed active site residues Glu 315 and Asp 391 are drawn as ball and stick models and coloured black.



(B) Ribbon diagram of ChiA drawn in an orientation to show the catalytic α/β barrel domain α-helices are coloured red, and β sheets are coloured in yellow.

Diagrams were produced from the crystal structure derived by Perrakis *et al.* 1994 (Brookhaven data base accession number 1ctn) using the software program RasMol via the WWW site at the Univ. of Massachusetts,

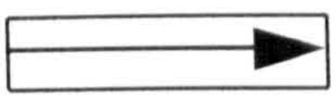
1.12.5 Organisation of domains in chitinases

Sequence analyses of bacterial chitinases have shown that these enzymes possess short regions of high sequence homology, despite the overall divergence of their primary structures. It is also apparent that the organisations of the functional domains of these proteins differ considerably between chitinases (figure 1.7) and that domain rearrangements, such as deletion, insertion or duplication of domains such as fibronectin type III domains and chitin binding domains may have arisen in the process of evolution (Gleave *et al.*, 1995).

Figure 1.7 Domain structure of various chitinases

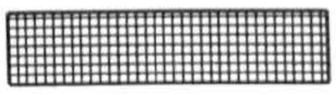
Key :-

—— Catalytic domain



—— Fibronectin type III- like domain

—— Chitin binding / putative chitin binding domain



—— Cellulose binding -like domain

40

1.13 Possible roles of chitinases in the transmission of parasites

Blood-sucking insects act as vectors for many disease-causing parasites; some parasites are simply carried between vertebrate hosts whereas others undergo obligatory morphological transformation or replication within the insect vector. In order to establish infections in their insect vectors, parasites have to negotiate their passage through the chitinous membranes of their host.

The production of chitinolytic enzymes by insect infectious agents has been shown to be important for *Serratia marcescens* (Flyg and Boman, 1988), *Plasmodium spp* (Huber *et al.*, 1991), *Leishmania spp.* (Schlein *et al.*, 1991), various nematodes (Fuhrman *et al.*, 1992; Harshbarger and Faust, 1973); trypanosomes, (Freeman, 1973; Ellis and Evans, 1977) and tsetse fly endosymbionts, (Maudlin and Welburn, 1988).

Table 1.1

Possible roles of chitinolytic enzymes in the transmission of parasites

ROLE	ORGANISM	REFERENCE
Penetration of peritrophic membrane	***Leishmania spp*** **Trypanosomes** ***Plasmodium spp.***	**Schlein *et al.* (1991)** **Ellis and Evans (1977)** **Huber *et al.*, (1991)**
Blocking of insect defensive lectins	***Brugia malayi*** **Tsetse endosymbionts**	**Fuhrman *et al.*, (1992)** **Maudlin and Welburn (1987)** **Welburn (1991)**
Ex-sheathment	***Brugia malayi***	**Fuhrman *et al.*, (1992)**
Cuticular breakdown of dead insect	***Xenorhabdus***	**Harshbarger and Faust (1973)**
Egg/Cyst hatching	***Onchocerca gibsoni***	**Gooday *et al.*, (1988)**

1.13.1 The peritrophic membrane barrier to parasite infection

In many species of insects the midgut tissue is separated from the bloodmeal by a peritrophic membrane (PM), defined as a membranous acellular sac surrounding the ingested food (Richards and Richards, 1977). Peritrophic membranes may be divided into two categories designated type I and type II (Ponnudurai *et al.*, 1988). Type I are produced from a diffuse secretory area over the complete length of the midgut, and this is the most common method for the production of the PM and is widespread in insects,

including sandflies, adult mosquitoes and blackflies. Type I PMs are discontinuously secreted with production normally initiated by the mechanical distension resulting from the uptake of the bloodmeal (Chiang and Davey, 1988). The formation then takes from 1-36 hours to mature depending on the insect species (Huber *et al.*, 1991; Miller and Lehane, 1993; Shahabuddin and Kaslow, 1993a). Consequently, parasites entering a vector producing a type I PM initially face little obstruction to their invasion of midgut epithelium. However, as time proceeds the obstruction becomes increasingly substantial.

In contrast, type II PMs are formed from a localised area called the cardium or proventriculus, situated at the junction between the fore and midgut of the insect. Type II PMs are found in all larval diptera and in adult tsetse, hibotoscids and biting muscids (Ponnudurai *et al.*, 1988). They are produced continuously and form permanent barriers between ingested parasites and the midgut epithelium. As implied above, a single insect species may produce different PMs at different developmental stages.

The temporal relationship between parasite development and PM maturation in insects producing type I PMs is fundamental to the parasite reaching and invading/damaging the midgut epithelium. The time taken before the PM matures is a window of opportunity for microfilariae in blackflies, as the parasite invades the midgut before PM maturation at 4 hours after feeding (Miller and Lehane, 1993). The vector-parasite specificity of different mosquitoes and different plasmodium species may also be explained by this balance between speed of parasite development (non-motile gametocyte to motile invasive ookinete) versus speed of PM formation (Ponnundurai *et al.*, 1988; Miller and Lehane, 1993). The PM would therefore appear to function as a rate limiting mechanism for some parasites and a total block for others, depending on the speed of development of the parasites. This was described by Sieber *et al.* (1991) who showed that the avian malaria parasite, *Plasmodium gallinaceum,* develops motile ookinetes in the blood meal 16-20 hours after their ingestion and then invade the midgut epithelial cells from 20-24 hours after feeding. However, the PM is fully mature at 12 hours and does not dissolve until at least 48 hours later. The parasites therefore have to traverse the PM to move from the bloodmeal to the epithelial cells of the mosquito midgut. This study also revealed ookinetes trapped in the endoperitrophic space during invasion and a lower number of oocysts in the midgut in comparison with the number of ookinetes in the endoperitrophic space; again, both observations suggested that the

peritrophic membrane acts as a barrier to infection. This interpretation has, however, been challenged by the results of Shahabuddin *et al.* (1993b), who found that disruption of the PM by feeding exogenous chitinase did not significantly increase or decrease the number of oocysts that formed per gut relative to controls. This finding indicates that the PM by itself does not limit the number of parasites that develop into oocysts. However, further direct evidence was provided in an ultrastructurel study which showed degradation of the PM adjacent to the penetrating ookinetes. It was concluded that the parasite does digest the PM as it passes through the membrane (Sieber *et al.*, 1991). Similar results were also recorded for *Babesia microti* passing through the PM of *Ixodes* ticks (Rudzinska *et al.*, 1982).

1.13.2 Involvement of chitinases in the transmission of *Plasmodium* spp.

The PM has been shown to be composed of chitin in mosquitoes, based on work by a number of authors. Maeda and Ishida (1967) suggested that the PM contains chitin, based on the high fluorescence of the membrane after exposure to Calcofluor white (a fluorescent brightener that has high affinity for chitin). Rudin and Heckler (1989) demonstrated that N-acetyl-D-glucosamine specific lectins bound to the PM of *Aedes aegypti*, and the findings of this experiment was verified by Huber *et al.* (1991) who also specifically demonstrated the presence of chitin in the PM by *invitro* digestion with *S. marcescens* chitinase to produce free N-acetyl-glucosamine.

The digestion of the PM by parasite enzymes was first postulated by Freyvogel and Jaquet (1965). Huber *et al.* (1991) demonstrated the presence of at least one chitinase in *P. gallinaceum* and demonstrated that this is synthesised and secreted *in vitro* at about the same time that the *P. gallinaceum* ookinetes would be penetrating the PM *in vivo*.

Evidence that chitinase activity is involved in the life cycle of *Plasmodium* has also come from experiments in which the competitive chitinase inhibitor, allosamidin, was added to the feed (Shahabuddin *et al.*, 1993b). Allosamidin was found to block completely the transmission of *P. gallinaceum* in *Aedes aegypti* and *P. falciparum* in *Anopheles freeborni*, when fed at a concentration of 0.1mM in infected blood. The possibility that allosamidin killed the parasite or otherwise interfered with normal sporogenic development by some mechanism other than inhibition of parasite chitinase was ruled out by incorporating exogenous *Streptomyces griseus* chitinase (an enzyme unaffected by allosamidin) as a control. The chitinase completely disrupted PM

formation, and reversed the transmission blocking effect of allosamidin. A cautionary note, however, is that allosamidin caused certain anomalies in PM formation; the membrane appeared earlier, thicker, and more disorganised, as well as persisting longer than in the control flies. This observation would not exclude the possibility that whatever caused allosamidin to thicken the PM was also responsible, in part at least, for its transmission-blocking activity.

1.13.3 Involvement of chitinases in the transmission of *Leishmania spp.*

Chitinase activity in the Trypanosomatidae was first reported by Schlein *et al.* (1991). Chitinases were found to be widely distributed in trypanosomatids, being secreted by:- *Leishmania major*, *L. braziliensis*, *L. donovani*, *L. infantum*, *Leptomonas seymouri*, *Crythidia fasciculata*, *Herpetomonas muscarum*, *Trypanosoma lewisi*, and *Trypanosoma brucei brucei* (Schlein *et al.*, 1992).

In a histological study, Schlein *et al.* (1991) observed that infections of *L. major* altered the normal pattern of disintegration of the PM of *Phlebotomus papatasi*. In uninfected flies, break down of the PM begins at the posterior end whereas in sandflies infected with *L. major* breakdown at the posterior end was preceded by breakdown along both sides as well as at the anterior end. The parasites accumulated at the anterior end of the PM and disintegration of the PM facilitated their forward migration to the fore gut and into the vicinity of the cardiac valve. It was concluded that this abnormal break-down of the PM was due to expression of chitinase by *Leishmania* promastigotes. The parasites were subsequently observed to concentrate in the cardiac valve region on the fourth day off infection and damage it. The cardiac valve is a prolongation of the fore gut which bulges into the stomach and has an internal layer of thin cuticle. The epithelial cells atrophy and their cuticular lining is lost; this damage culminates on the 14th day off infection, with epithelial cells reduced to half of their original size and the normal smooth outer surface corrugated with angular protrusions on individual cells. The overall result is valve dysfunction and an increased vectorial capacity for the sandfly. Evidence was later provided to suggest that the damage to the cardiac valve can be prevented by conditions that inhibit the secretion of chitinases; the chitinase system of *L. major* was found to be inhibited by the presence in culture media of both blood and haemoglobin (Schlein *et al.*, 1991, 1992 and Schlein and Jacobsen 1993). Furthermore, the authors compared the condition of the cardiac valve with the feeding performance of three series of flies, namely-

(1) blood-fed infected (non-transmitting) flies,

(2) sugar-fed infected (transmitting) flies,

(3) uninfected flies

Damage to the cardiac valve was observed in infected sugar-fed flies; however the cuticular cover remained intact in infected flies fed repeatedly with blood. The authors suggested that the haemoglobin in the sandfly bloodmeal suppresses the secretion of chitinases by the parasites until it has been digested. This finding agrees with the observation of Sacks and Perkins (1985) who noted that the elimination of the sandfly's bloodmeal is concurrent with a sudden increase in promastigote infectivity and lysis of the PM. Schlein (1991) reports further evidence that damage to the cardiac valve impairs the process of feeding in a manner that can potentiate transmission of *Leishmania* parasites by bite.

1.13.4 Involvement of chitinases in the transmission of nematodes

Brugia malayi is a parasitic nematode that affects nearly 100 million people world-wide, causing the disease lymphatic filiariasis (Fuhrman *et al.*, 1992). Transmission of the disease depends on the ability of the parasite to persist in the peripheral blood of its vertebrate host, as well as its capacity to infect and develop in its insect vector, the mosquito. Canlas *et al.* (1984) found that a monoclonal antibody (MF1) recognised two stage-specific proteins, P70 and P75, and that this antibody mediated clearance of microfilaria in the peripheral blood of a gerbil animal model. The appearance of P70 and P75 in microfilaria coincided with the onset of infectivity of the parasite for its insect host (Fuhrman *et al.*, 1987). Furthermore, cDNA prepared from mRNA isolated from the parasite using degenerate primers, designed against partial amino acid sequences of P70 and P75, showed primary sequence homology to a number of cloned chitinase genes. The P70 and P75 proteins were also shown to have chitinase activities against glycol chitin in SDS PAGE overlay gels. The possibility that these chitinases function to degrade the PM in the mosquito is questioned by the fact that the parasite invades mosquito midgut epithelial cells within an hour of uptake in the bloodmeal, which is before the PM forms (Sutherland *et al.* , 1986). Fuhrman *et al.* (1992) suggest two possibilities for the involvement of the chitinase in parasite transmission :-

(1) It could be involved directly in the degradation of chitin-containing structures of the microfilaria. Female adults of *Brugia spp.* shed live microfilaria, which retain a remnant of their eggshells called the extracuticular sheath. Exsheathment occurs during the initial development of the filarial larvae in the mosquito vector; thus the chitinase

appears at the same time as infectivity.

(2) The chitinase could be involved indirectly in blocking an insect defence mechanism. The chitinase could generate inhibitory oligosaccharides and promote infection by blocking a mosquito defensive lectin. The possibility of such a lectin has been suggested by Phiri and Ham (1990) who demonstrated that feeding N-acetylglucosamine in the infective bloodmeal blocked microfilarial invasion of the midgut epithelium.

Chitinase activity has also been reported in *Onchocerca gibsoni,* the infective agent of cattle filariasis (Gooday *et al.*, 1988). In this filarial species, the activity is present in the adult female. *Onchocerca gibsoni* may use its chitinase in the remodelling of chitin during egg development in the vertebrate host and hence this enzyme activity may also be essential for its transmission (Shahabuddin *et al.*, 1993).

Chitinase activity has been implicated in the transmission of nematodes belonging to the families Steinernematidae, Heterorhabditidae and Neoaplectana (Harshbarger and Faust, 1973). In these cases, the chitinase is secreted by symbiotic bacteria associated with the nematode. The bacteria are carried in the gut of the nematode, and are released into the insect after the nematode has gained entry to the insect. The entomopathogenic bacteria destroy the insect within 48 hours and the chitinase is believed to breakdown the insect carcass, thereby releasing the nematode offspring and providing nutrients for their growth.

1.13.5 Chitinase involvement in the transmission of trypanosomes

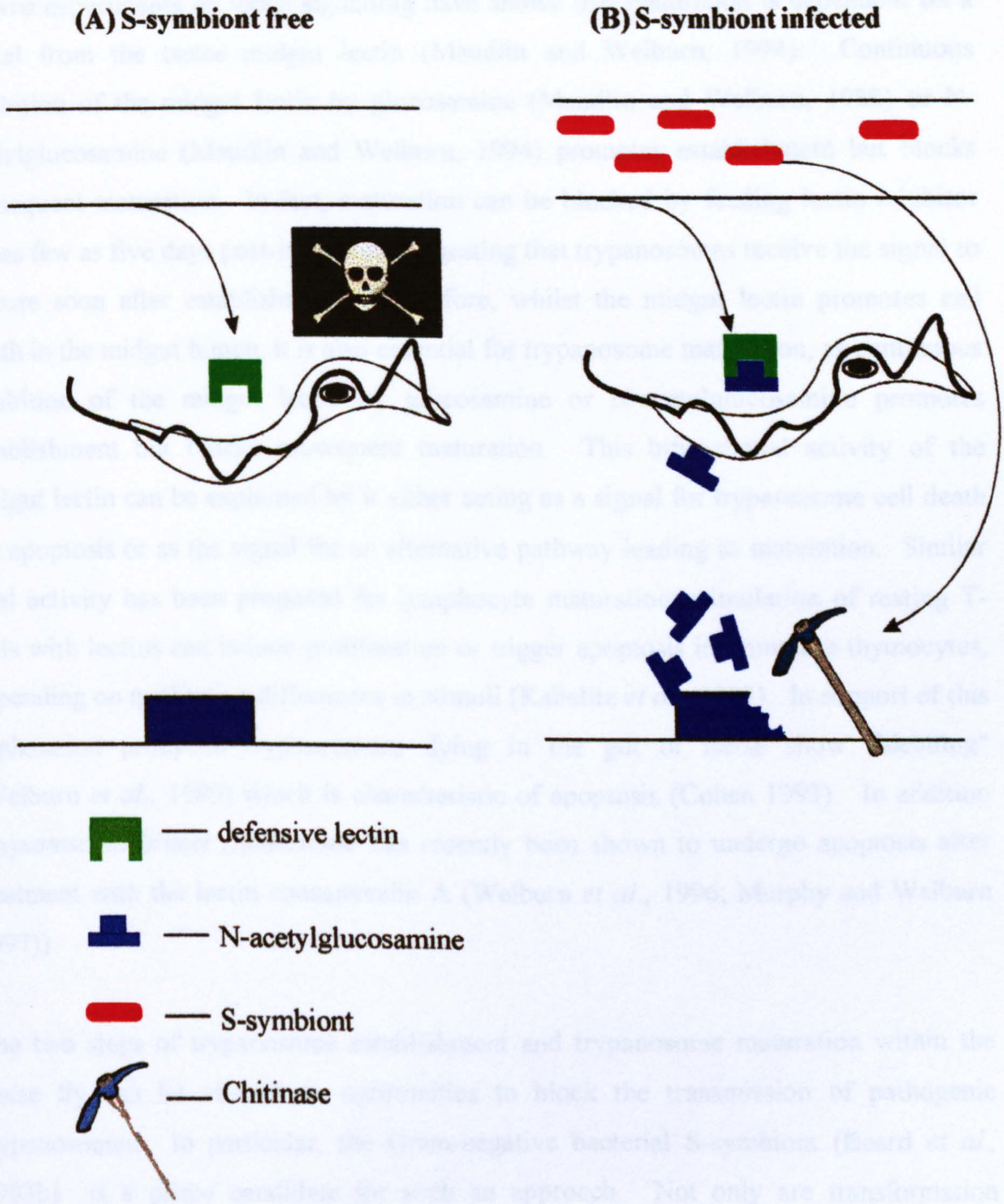
The tsetse fly, the major vector of pathogenic trypanosomes, has a type II PM that is always present. Therefore, pathogenic trypanosomes have either to bypass this barrier or penetrate it in order to migrate to the midgut epithelium and invade the fly. Ellis and Evans (1977) presented evidence that trypanosomes do indeed penetrate the PM; they observed penetration throughout the central two thirds of the PM and this was related to trypanosome concentration which was greatest in these central sections. Furthermore, penetration was found where the PM separated into two layers and this separation was only found in the presence of trypanosomes. Digestive enzymes were implicated as playing an important role in this process. Similarly, Freeman (1973) also provided evidence that trypanosomes pass across the PM.

In addition to the observations above, S-symbiont chitinase has also been implicated in transmission of trypanosomes by tsetse. Welburn *et al.* (1993) detected high endochitinase activity in the culture medium of this tsetse bacterial symbiont, called a Rickettsia-like-organism (RLO) in most of the literature because of its intracellular nature, although more recently it has been placed among the enterobacteriacea based on 16S rRNA phylogenetic analysis and is generally termed "secondary symbiont" (S-symbiont) to distinguish it from the tsetse primary endosymbiont (P-symbiont) (Beard *et al.*, 1993b see also chapter 3, this thesis). Chitinase activity in the midguts of tsetse was studied using fluorescent chitinase substrates; *Glossina morsitans morsitans,* a tsetse species known to have a high carriage of S-symbionts and to be highly susceptible to trypanosome infection, was shown to have higher endochitinase activity than a refractory species, *Glossina austeni*, which has a low level of S-symbiont infection (Welburn and Gibson, 1989) The endochitinase was also demonstrated to be distinct from chitinases produced by host culture cells.

Evidence is accumulating that the S-symbiont chitinase is responsible for rendering tsetse susceptible to infection with pathogenic trypanosomes. It is believed that this bacterial chitinase acts indirectly in this process, in a similar manner as has been suggested for *Brugia malayi*, by producing oligosaccharides that block a tsetse defence system based on a midgut lectin (see figure 1.8). Ibrahim *et al.* (1984) have demonstrated the presence of a lectin in the tsetse midgut which can be specifically inhibited by D-glucosamine. Experiments involving feeding D-glucosamine and N-acetyl glucosamine to flies significantly increased midgut infection rates (Maudlin and Welburn, 1987; Welburn, 1991), indicating that this could well be happening naturally. S-symbionts also show the same mode of extrachromosomal inheritance as susceptibility to infection with trypanosomes (Maudlin and Ellis, 1985) and are probably passed from one generation to the next through the milk gland secretions in the uterus and hence invade the midgut of the developing larvae (Bonnanfant-Jais, 1974). It has also been shown that susceptibility of flies to infection with *Trypanosoma congolense* and *Trypanosoma brucei* is a maternally inherited characteristic (Maudlin *et al.*, 1986) and in studies of both laboratory-reared and wild flies this has been associated with the presence of the intracellular S-symbionts. Moreover, susceptibility to trypanosome infection is quantitatively related to S-symbiont load in teneral *G. m. morsitans* (Welburn and Maudlin, 1992). Susceptibility to trypanosome infection has also been shown to be a strictly teneral phenomenom (Buxton, 1955; Maudlin and

Dukes, 1985; Maudlin and Welburn, 1992) in that flies must receive infected blood at their first bloodmeal after emerging from the puparium to become infected. It is concluded therefore, that S-symbionts in non-teneral flies have insufficient time between meals to produce sufficient inhibitory oligosaccharides to influence infectability, especially since there is good evidence that bloodmeal serum activates the secretion of midgut lectin (Gingrich *et al.*, 1982; Stiles *et al.*, 1990; Maudlin and Welburn, 1992). However, S-symbiont numbers increase rapidly during larval/pupal development in tsetse (Welburn, 1991); (Dr Ian Maudlin personal communication) and the enhanced susceptibility of the teneral fly would be expected via the action of bacterial chitinase producing inhibitory oligosaccharides during pupation that block the tsetse lectin-based defense system.

Figure 1.8 Model of generation of susceptibility to trypanosome infection in tsetse



In addition to establishing infections in the midgut of the tsetse, trypanosomes have to mature subsequently into mammalian infective forms, move to the mouthparts (*T. congolense*) or salivary glands (*T. brucei*) and transform to dividing epimastigotes and finally metacyclics, before the infection can be passed on to the next mammalian host.

In vivo experiments on lectin signalling have shown that maturation is dependent on a signal from the tsetse midgut lectin (Maudlin and Welburn, 1994). Continuous inhibition of the midgut lectin by glucosamine (Maudlin and Welburn, 1988) or N-acetylglucosamine (Maudlin and Welburn, 1994) promotes establishment but blocks subsequent maturation. In fact, maturation can be blocked by feeding lectin inhibitor for as few as five days post-infection, suggesting that trypanosomes receive the signal to mature soon after establishment. Therefore, whilst the midgut lectin promotes cell death in the midgut lumen, it is also essential for trypanosome maturation, as continuous inhibition of the midgut lectin by glucosamine or N-acetylglucosamine promotes establishment but blocks subsequent maturation. This bifunctional activity of the midgut lectin can be explained by it either acting as a signal for trypanosome cell death via apoptosis or as the signal for an alternative pathway leading to maturation. Similar dual activity has been proposed for lymphocyte maturation; stimulation of resting T-cells with lectins can induce proliferation or trigger apoptosis in immature thymocytes, depending on qualitative differences in stimuli (Kabelitz *et al.*, 1993). In support of this explanation procyclic trypanosomes dying in the gut of tsetse show "blebbing" (Welburn *et al.*, 1989) which is characteristic of apoptosis (Cohen 1993). In addition *Trypanosoma brucei rhodesiense* has recently been shown to undergo apoptosis after treatment with the lectin concanavalin A (Welburn *et al.*, 1996; Murphy and Welburn 1997))

The two steps of trypanosome establishment and trypanosome maturation within the tsetse fly can be viewed as oportunities to block the transmission of pathogenic trypanosomes. In particular, the Gram-negative bacterial S-symbiont (Beard *et al.*, 1993b) is a prime candidate for such an approach. Not only are transformation techniques and vectors well developed for Gram-negative bacteria, but the presence of the S-symbiont within the midgut of the tsetse fly ideally suits the S-symbiont for production of transmission blocking proteins/metabolites. This pseudotransgenic approach to produce tsetse incapable of transmitting pathogenic trypanosomes and the subsequent diseases that they cause can be achieved in a number of ways; one tsetse

group based at Yale University USA is hoping to express a gene from a mammalian immune system to block trypanosome transmission (Aldhous, 1993). However a more subtle approach would be to either manipulate chitinase activity in such a way as to block maturation of trypanosome infections or increase the utilisation of D-glucosamine and N-acetyl glucosamine by overexpression of a permease gene in the S-symbionts. Both scenarios have the probability of rendering the tsetse fly resistant to trypanosome infection. As a first step in this process this thesis was designed to characterise the chitinase system of this bacterium and to clone genes which could be used to eventually produce pseudotransgenic tsetse that are incapable of transmitting disease.

Section 2

Materials and Methods

2.1 Isolation of tsetse secondary symbionts from *Glossina* spp. and their culture in *Aedes albopictus* cells

'Secondary endosymbionts' (S-symbionts) were obtained from the haemolymph of 20 newly emerged tsetse. Flies were surface sterilised for 20 minutes in three changes of Alcide L.D. (Life Science Laboratories), their heads were punctured in the fons region and haemolymph was collected in sterile 2 µl micropipettes (Camlab). The pooled haemolymph was subsequently pipetted into 100 µl of Mitsuhashi and Maramorosh Basal medium supplemented with 20% v/v foetal calf serum (M&M culture medium Flow laboratories) and divided between three flat bottom centrifuge tubes containing circular coverslips previously seeded with *Aedes albopictus* cells (Flow Laboratories) and incubated at 26°C for 5 days. The secondary symbionts divide in the cytoplasm of the *A. albopictus* cells causing widespread cell lysis after 96 hours, resulting in release of these organisms from the cells into the growth medium. To separate symbionts from host cells, remaining intact host cells in the culture were removed by low speed centrifugation (500 xg for 10 min) leaving free symbionts in the supernatant. The supernatant was then centrifuged (20,000 xg at 4°C for 25 mins) to pellet the bacterial cells, the pellet was then resuspended in 100ul phosphate buffered saline (PBS) pH 6.8 and used to inoculate 100ml cell culture flasks previously seeded with *A. albopictus* cells. Cultures were routinely checked for growth of S-symbionts by staining using the Gimenez staining method (Gimenez, 1964). S-symbionts observed as red organisms within the host cell cytoplasm were then re-passaged by inoculation onto further confluent *A. albopictus* monolayers as before. Routine passage was performed at weekly intervals.

2.2 Culture of secondary symbionts on blood agar plates

S-symbionts were also grown on blood agar plates. *Aedes albopictus*/symbiont culture (section 2.1) was resuspended by scraping the cells from the bottom of the culture flask. The symbionts were then isolated by differential centrifugation and resuspended as above in phosphate buffered saline. A 100µl portion of S-symbionts was then spread on a sheep blood agar plate (section 2.4.6). The plate was placed in a gas-tight jar, and the air was replaced with a gas mixture of 5%(v/v) oxygen 10%(v/v) carbon dioxide and 85% (v/v) nitrogen. The gas jar containing the blood agar plate(s) was then placed in an incubator at 26°C for 3-4 days. Symbionts were then routinely subcultured twice weekly on blood-agar plates.

Blood agar plates were prepared by adding 5ml of sterile defibrinated sheep blood (Tissue Culture Services Microbiology Limited, Buckingham UK) to 95 ml of sterile nutrient agar at 50°C. This was sufficient to prepare five plates.

2.3 Biochemical tests

Biochemical tests were performed as described by MacFaddin 1976.

The catalase test was performed using the slide method : the centre of a pure colony of the bacteria was picked with an inoculating needle and placed on a clean glass slide. A single drop of 30% hydrogen peroxide solution was then added to the bacteria. Immediate bubbling indicates the presence of the enzyme catalase. A colony of *E. coli* was used as a positive control.

The oxidase test utilised Kovac's oxidase reagent (1-% tetramethyl-*p*-phenyllenediamine dihydrochloride. 2-3 drops of the Kovac's reagent were added to the centre of a piece of whatman No. 1 filter paper in a petri dish. A loopful of the suspected colony was then smeared onto the impregnated paper in a line 3-6 cm long. Positive results are indicated by a colour change within 5-10 seconds.

The nitrate reduction test used two reagents: α-naphthylamine (0.5% in 30% acetic acid) and sulfanilic acid (0.8% in 30% acetic acid). Semi solid medium (0.3% Nitrate agar purchased from Difco) was inoculated with a large loopful of test bacteria. Positive and negative controls consisted of additional tubes of agar inoculated with *E. coli* and *Acinetobacter calcaoceticus* respectively. The cultures were incubated and then 1ml of each reagent was added. A positive result is indicated by a red colour development in 30 seconds.

Indole production was tested using Kovac's indole reagent (150ml isoamyl alcohol, 10g p-Dimethylaminobenzaldehyde, 50ml concentrated HCl) and Difco tryptone broth. The broth was inoculated with the test organism and *E. coli* was used as a positive control. The cultures were incubated and then 5 drops of Kovac's reagent was added directly to the tubes. A positive result is indicated by a red colour in the alcohol layer.

The Voges-Proskauer test consisted of innoculating semi solid Voges-Proskauer medium (Tryptone 10g, yeast extract 5g, NaCl 5g, K_2HPO_4 5g, Glucose 5g and agar 3g in 1L water) with a large loopful of test bacteria. The cultures were then incubated and then 1 drop of creatinine solution (1% creatinine in 0.1M HCl) and 0.5ml of a freshly prepared mixture of three parts 5% naphthol (in 100% ethanol) and one part 40% KOH were placed on the surface of the agar. The test was then read after 1h. A positive result is indicated by a red colour development. Positive and negative controls consisted of

tubes innoculated with *Enterobacter clocae* and *Escherichia coli* respectively.

The Urease test used Christensen's agar. Slants of the agar were inoculated with the test bacteria and incubated. A positive result consists of a red-pink colour change through the medium.

The hydrogen sulphide test utilised Kliger's iron agar (Difco). Briefly slants of Kligers iron media were inoculated with the test organisms by smearing the surface of the slant and then stabing the medium. Blackening of the media indicates a positive result.

Gelatin liquefaction was tested by inoculating a tube of gelatin stab medium (Difco) with the test organism.

Liquid cultures were incubated at 26°C; cultures on agar media were incubated in an atmosphere of 5% oxygen, 10% carbon dioxide and 85% nitrogen at 26°C. Cultures were left for 14 days and checked twice weekly for test results. Tests of acid production from various sugars were performed by inoculating individual 4.5ml broths of Mitsuhashi and Maramorosh medium pH 7.4 containing 0.5% (w/v) concentration of the respective sugar with 0.5ml of S-symbiont culture. Acid production was scored by colour change, red-yellow, of phenol red indicator, against a control containing no sugar. The broths were scored every day for 3 days.

2.4 Bacterial strains and Plasmids

2.4.1 *E. coli* strains

The *E. coli* strains used in this study were :-

:Strain	Genotype	Reference/Source
DH5α	*F- end A1, gyrA96, hsdR17(rk^-, mk^+) supE44 thi-1 rec A1 rel A1, Δ(argF - lacZYA)U169 (ϕ80d lacZ M15)*	Hanahan, (1983)
JM109	*recA1, endA1, gyrA96, thi, hsdR17 (rk^-,mk^+), supE44 Δ(lac-pr0AB), relA1, [F', traD36, proAB, lacI^{q}ZΔM15]*	Yanisch-Perron, (1985)
LE392	*F', hsdR574, (rk^-,mk^+), supE44, supF58, Δ(lacIZY)6, galK2, galT22, metB1, trpR55*	Murray *et al*, (1977)
LR2-167	*F-phoA, thi1, argB6, metB, his1, galT, rpsL, manA, nagE.*	Postma & Lengeler, (1985)

E. coli strains JM109 and LE392 were purchased from Promega Biological Research Products, Madison, USA. LR2-167 was a gift from Dr Bruce Waygood, University of Saskatchewan, Saskatoon, Canada

2.4.2 S-symbiont strains

Glossina morsitans morsitans secondary symbiont was isolated from *Glossina morsitans morsitans* (Westwood)

Glossina palpalis palpalis secondary symbiont was isolated from *Glossina palpalis palpalis* (Robineau-desvoidy)

Glossina austeni secondary symbiont was isolated from *Glossina austeni* (Newstead)

All species of secondary symbiont were isolated from flies obtained from populations of established colonies, at the Tsetse Research Laboratory, Langford, Bristol.

2.4.3 The plasmids used in this study were:-

Plasmid	Description
pUC18	cloning vector encoding resistance to ampicillin (Yanisch-Perron *et al,* 1985).
pGem-3Z	cloning vector encoding resistance to ampicillin
pGem-3Z(Kan)	derivative of pGem-3z containing 1.2kb kanamycin gene from Tn*902* in addition to the original marker.
pKP1.1	derivative of pUC19 containing a 5.9kb *Bgl*1 fragment of *E. coli* chromosomal DNA, coding for enzyme II $^{\text{N-acetylglucosamine}}$ of the PTS system
pDD509	derivative of plasmid containing gentamycin gene (Stanley *et. al.*, 1989)
pYZ291	Derivative of plasmid containing *S. marcescens chi*A (Sitrit *et. al.*, 1993)
pCD5.13	pBluescript SK+ containing a 1.8kb *pst*1-*Hin*dIII fragment from an extrachromosomal element from S-symbiont (*G. m. morsitans*). Encoding an helicase 1 homologue.
pMAT1	pUC18 containing 5.2 kb of symbiont chromosomal DNA encoding the N-terminus of the chitinase. (This study)
pMAT1a-e	pUC18 containing various fragments of the insert of pMAT1. (This study)
pMAT1.1-1.8	Small cloned fragments of pMAT1 used for sequencing the pMAT1 insert (This study)
pMAT1DB	*Bam*H1 1.6kb deletion of pMAT1. (This study)
pMAT1DS	*Sal*1 1.6kb deletion of pMAT1. (This study)
pMAT1DSm	*Sma*1 2.8kb deletion of pMAT1. (This study)
pMATa22	pUC18 containing 1.4kb of symbiont chromosomal DNA encoding the C terminus of the chitinase. (This study).
PMAT2	*Hin*dIII deletion of pMATa22. (This study)
pMAT3	pUC18 containing 4.7kbp of symbiont DNA encoding the entire chitinase gene. (This study)
pXylHD	507 bp *pvu*II/*Kpn*1 fragment of the xylose-H^+ gene of *E. coli* cloned into the *Sma*1 site of pUC18
pRSF1010	incQ broad host range vector, encoding resistance to streptomycin and sulfonamides. (Scholz *et al.*, 1989)

Plasmid pUC18 was purchased from Pharmacia, pGEM-3Z and λ genomic cloning vector LambdaGEM-11 were purchased from Promega. Plasmids pKP1.1, pDD509, pCD5.13 and pYZ291 were gifts from Dr Bruce Waygood (University of Saskatoon, Canada), David Dowling (University of Cork, Ireland), Colin Dale (University of Glasgow) and Professor Oppenheim (Rehovat University, Israel), respectively. Plasmid pXylHD was constructed by cloning a fragment of the xylose-H^+ gene (encompasing the region of DNA coding for the PESPRWL and TVDKA/FGR motifs) supplied by Professor Henderson, University of Leeds, into the *Sma*1 site of pUC18.

2.5 Chemicals and reagents

Unless stated otherwise chemicals were purchased from BDH Chemicals Ltd. or from Sigma Chemical Co. Ltd. Media constituents were obtained from Difco Laboratories. Radiolabelled chemicals, and random priming kits were supplied by Amersham. Enzymes, together with commercially supplied buffers, were obtained from either Gibco (BRL) or Pharmacia Ltd.

Synthetic chitin substrates 4-methylumbelliferyl-N,N',N'',N'''-tetra-acetyl-β-D-chitotetraoside ($4MU(GlcNAc)_4$), 4-methylumbelliferyl-β-D-N,N',N''-triacetylchitotrioside ($4MU(GlcNAc)_3$), 4-methylumbelliferyl-β-D-N,N',-diacetylchitobioside ($4MU(GlcNAc)_2$) 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (4MUGlcNAc), p-nitrophenyl N-acetyl-β-D-glucosaminide (PNPGlcNAc), p-nitrophenyl-β-D-N,N'-diacetylchitobiose ($PNP(GlcNAc)_2$) and p-nitrophenyl-β-D-N,N',N''-triacetylchitotriose ($PNP(GlcNAc)_3$) were all purchased from Sigma Chemical Company Ltd. Stock solutions of synthetic chitin substrates were made up to 5mg.ml^{-1} in di-methyl sulphoxide (DMSO)

2.6 Growth Media.

2.6.1 Nutrient broth.

LabM nutrient broth No.2 was made up according to the manufacturer's instructions (Oxoid Ltd.).

2.6.2 Nutrient agar. Nutrient agar was made from blood agar base (Lab M) according to the manufacturer's instructions (Oxoid Ltd.).

2.6.3 Minimal salts medium (x2).

K_2HPO_4	14.0g
KH_2PO_4	6.0g
$(NH_4)_2 SO_4$	2.0g
$Na_3C_6H_5O_7.2H_2O$	1.0g
$MgSO_4.7H_2O$	0.5g

Made up to a total volume of 1 litre with distilled water.

This was mixed with an equal volume of 3.3% molten agar for solid medium; for liquid medium, an equal volume of distilled water was added. In both cases the minimal salts medium was supplemented with 1/100vol of 40% (w/v) glucose and growth supplements, as required.

2.6.4 LB broth

Bacto-tryptone (Oxoid)	10g
Bacto-yeast extract (Oxoid)	5g
NaCl	10g

Made up to a total volume of 1 litre with distilled water.

2.6.5 SOC.medium

Bacto-tryptone (Oxoid)	20.0g
Bacto-yeast extract (Oxoid)	5.0g
NaCl	0.58g
KCl	0.19g

Made up to a total volume of 1 litre with distilled water. The broth was then supplemented with $MgCl_2$, $MgSO_4$, and glucose to final concentrations of 10mM, 10mM and 20mM, respectively.

2.6.7 Minimal media for mutational complementation of LR2-167

Minimal agar was prepared as described in 2.6.3 except that it contained N-acetylglucosamine instead of glucose and was supplemented with 10ml per litre of 10mg.ml^{-1} solutions of arginine, methionine, histidine and thiamine.

2.7 Sterilisation of Media.

Media were sterilised by autoclaving at 0.7kg cm^{-2} for 20min at 121°C. If the media contained thermolabile constituents, such as glucose, autoclaving was carried out at 0.35kg cm^{-2} for 20min.

2.8 Antibiotics.

Stock solutions of chloramphenicol 30mg.ml^{-1} and tetracycline 25mg.ml^{-1} were prepared in 70%(v/v) ethanol and stored at -20°C. Streptomycin, kanamycin, ampicillin and streptozotocin were made up as 100mg ml^{-1} or 25mg ml^{-1} stock solutions in distilled water, filter sterilised and stored in aliquots at -20°C. Nalidixic acid was prepared in 1mM NaOH as a 25mg ml^{-1} stock solution, filter sterilised, and stored at -20°C.

2.9 Transformation Procedures.

2.9.1 Routine transformation using $CaCl_2$.

The method used was from Current Protocols in Molecular Biology Vol.1 section 1.8.2, (Ausubel *et al.*, 1990a).

2.9.2 Preparation of electro-competent cells.

When a high frequency of recombinant plasmid recovery was required, i.e. for genome library construction, electrotransformation (electroporation) was used. 1ml of an overnight culture was added to 500ml prewarmed broth (with appropriate antibiotic) and the culture was incubated with shaking at 37°C until mid-logarithmic phase (Optical density of 0.6 at 600nm) had been reached. Cells were harvested by centrifugation at 4°C, 4,000rpm for 15min (Beckman J2-21, JA-10 rotor) and then suspended in 10ml ice-cold sterile distilled water. The suspension was diluted to 200ml with ice-cold water and the cells pelleted again as described previously. Cells were washed twice more in water, as described above. The thoroughly washed cells were resuspended in an ice-cold solution of 10% (v/v) glycerol and repelleted before finally being suspended in an equal volume of ice-cold 10% (v/v) glycerol. This gave a final suspension of 5×10^{10} cells ml^{-1}, which was aliquoted and stored at -70°C.

2.9.3 Transformation.

10-100ng of DNA were added to 50µl of competent cells and the mixture was left on ice for 1h, to ensure maximum binding of DNA to the cell surface. The cells were then heat shocked by transferring the mixture to a 42°C waterbath for 1min, which allows the DNA to enter the cells. The mixture was then added to 10ml of pre-warmed LabM broth and incubated for 1hour at 37°C with gentle agitation, to allow expression of introduced DNA in the cells. 200µl of culture was plated onto medium containing the appropriate antibiotic and plates were incubated overnight at 37°C. Transformation efficiencies of 10^6 transformants/µg of DNA were routinely achieved.

2.9.4 Electrotransformation.

1-2µl (10-100ng) of micro-dialysed DNA were added to a chilled electroporation cuvette. 40µl of electrocompetent cells were then added and mixed thoroughly with the DNA. The outside of the cuvette was wiped dry and the cuvette lightly tapped to remove all air bubbles and ensure the sample was evenly distributed over the bottom of the cuvette. One pulse was applied (Gene Pulser Biorad, 2.5kV, 25µFD, 400Ohm) and 1ml of pre-warmed (37°C) SOC medium was added immediately. Electroporated cells were transferred to a 1.5ml Eppendorf tube and incubated at 37°C for 20min. After incubation, 10-200µl portions were spread onto selective agar. Transformations efficiencies of 10^8-10^9 per ug DNA were routinely achieved with this method.

2.10 Preparation of Nucleic Acids.

2.10.1 Preparation of chromosomal DNA.

E. coli chromosomal DNA was prepared according to the method described in section 2.4.3 of Current Protocols in Molecular Biology (Ausubel *et al.*, 1990a). A CTAB extraction for removal of polysaccharides and protein was routinely included.

2.10.2 Preparation of DNA from secondary symbionts (A)

A washed pellet of the S-symbiont (prepared as described in section 2.1) was resuspended in 1ml of lysis buffer (10mM Tris, 1.25mM EDTA; 0.05%(v/v) SDS, pH 8 and heated to 65°C for 15 min before being placed on ice for 1 minute . Proteinase K was added to a final concentration of 100 $\mu g.ml^{-1}$ and the lysate incubated at 37°C for 30 minutes. The lysate was extracted twice with phenol and the DNA was precipitated

with alcohol (Maniatis *et al.*, 1982) The DNA was finally dissolved in sterile distilled water.

2.10.3 Preparation of S-symbiont DNA (B)

This method was used following the development of a culture system for the S-symbiont on blood agar under reduced oxygen conditions. An individual colony was streaked out on to blood agar and the plate was incubated at 28^{0}C for 3-4 days. The cells were then scraped off into 2 ml of nutrient broth, centrifuged to pellet them and then the DNA was isolated as described in section 2.4.3 of Current Protocols in Molecular Biology (Ausubel *et al.*, 1990a).

2.10.4 Preparation of S-symbiont Plasmids

The S-symbiont plasmids were isolated using Wizard Minipreps DNA purification system (Promega) according to the manufacturer's instructions; alternatively they were isolated by the method below.

2.10.5 Mini prep method

Mini preparations of plasmid DNA were made according to the method of Birnboim and Doly (1979), as described in Unit 1.6 of Short Protocols In Molecular Biology (Ausubel *et al.*, 1990b)

2.10.6 Maxi prep method

Maxi Preparations of plasmid DNA were prepared using Wizard Maxipreps DNA purification system (Promega) according to the manufacturer's instructions.

2.10.7 Purification of DNA by CsCl-Ethidium Bromide isopycnic centrifugation.

Caesium chloride gradients were prepared by dissolving 3.7g CsCl in 3.5ml of DNA solution. The solution was transferred to an ultracentrifuge tube containing 200µl of a 10mg ml^{-1} solution of ethidium bromide (EtBr). The tube was then filled to the neck with a 1g ml^{-1} solution of CsCl. Tubes were sealed and centrifuged at 48,000 rpm at 16°C for eighteen hours (Sorvall OTD65B, TV-865). DNA bands were detected by long wave UV illumination and their positions in the tube marked with a marker pen. A breather needle was inserted into the neck of the tube. Upper DNA (chromosomal) bands were removed by syringe fitted with a large gauge needle which was inserted into the side of the tube 0.5cm below the band. The lower (plasmid) bands were removed by

syringe fitted with a narrow gauge needle, again inserted immediately below the desired band. EtBr was removed from the DNA solution by repeated washes with CsCl-saturated isopropanol. The solution was then dialysed against 2L of 1mM Tris buffer, pH8, for 24h.

2.11 Enzymic Manipulation of DNA.

2.11.1 Restriction endonuclease digestion.

Where complete digestion of DNA was required, restriction digests were commonly set up as follows:

DNA	0.2µg-2µg
Buffer (x10)	1µl
restriction enzyme	1µl (2U)
sterile, distilled water to a final volume of 10µl	

The mixture was incubated at the temperature recommended by the supplier for 1 hour. If the restricted DNA was to be visualised on an agarose gel without further treatment, the reaction was terminated by the addition of 3µl stop mix (40% (w/v) sucrose, 0.2M EDTA pH8.0, 0.15% (w/v) bromophenol blue); otherwise reactions were terminated using a phenol/chloroform/isoamyl alcohol wash [25:24:1(v/v)] or by heating the mixture to an appropriate temperature for 10min.

2.11.2 Partial endonuclease digestions

Partial endonuclease digestion was achieved by either of two methods

(1) A pilot reaction was performed to determine optimal reaction conditions. Serial dilutions of the enzyme(s) were prepared and added to the reaction mixtures. Samples were withdrawn at set time intervals and the products analysed by agarose gel electrophoresis. The restriction was then repeated using those conditions which produced the desired partial digestion, scaling up as required; Or:-

(2) Ethidium bromide was added to the reaction mix to limit the number of sites cut by the enzyme. This involved setting up several reactions with ethidium bromide (100µgml^{-1}) added to give final concentrations from 5µgml^{-1} to 20µgml^{-1}. Digests were incubated at 37°C for 45 minutes.

2.11.3 Prevention of vector DNA from self-annealing.

Self-annealing of linearised vector during ligation reactions was prevented by pretreatment with calf intestinal alkaline phosphatase (CIP) which cleaves terminal phosphate residues. 5µg of vector DNA was first restricted with the appropriate restriction enzyme in a total reaction volume of 50µl. 10µl of CIP buffer was then added, together with 1µl (10U) CIP and 39µl water and the reaction allowed to continue for another 15min at 37°C. The reaction was terminated by a phenol/chloroform extraction. The DNA was then precipitated by the addition of 0.1 vol sodium acetate and 0.6 vol propan-2-ol. The dephosphorylated vector was then dissolved in 20µl sterile, distilled water to a concentration of $0.25\mu g.\mu l^{-1}$.

2.11.4 DNA Ligation.

Ligation of DNA was performed using T4 DNA ligase with the supplier's buffer. The total reaction volume was 25µl and 1µg of vector DNA was used. For subcloning experiments, a 3:1 molar ratio of insert DNA to vector was used. Ligation reactions were incubated overnight at 16°C. The ligation mix was heated at 70°C for 10min and micro-dialysed on filter discs (0.025µm pore; Millipore) for 30min prior to electotransformation.

2.12 Electrophoresis of DNA.

2.12.1 Agarose gel electrophoresis.

In most cases, horizontal agarose gel electrophoresis was used to size fractionate DNA fragments. Concentrations of Ultrapure™ agarose (BRL) used to make the gels varied from 0.7%-2%, depending on the size of the DNA fragments to be separated. Both standard and mini-gel electrophoretic apparatus were supplied by BRL. The buffer of choice for size fractionation of DNA was TBE buffer (0.09M Tris base, 0.09M boric acid and 2mM EDTA, pH 8.0). EtBr was added to a final concentration of $0.1\mu g\ ml^{-1}$ in both the gel and buffer. DNA patterns were visualised on a UV transilluminator and recorded photographically with a Polaroid CU-5 camera. Standard markers were made by cleaving λ DNA with either *Bgl*I, *Cla*I, *Eco*RI or *Hin*dIII restriction enzymes to obtain fragments with an optimal size range, or by using commercially prepared markers such as the 123bp ladder and 1kb ladder purchased from Gibco.

2.13 Isolation of DNA fragments from agarose gels.

Gel slices were excised from the agarose gel using a No11 scalpel blade. The gel slice was transferred to a dialysis tube and the DNA recovered by electroelution as described in section 2.6.1 of Current Protocols in Molecular Biology (Ausubel *et. al.*, 1990). Recovery of DNA by electroelution ensures a high % yield whilst not causing unnecessary shearing to the ends of the DNA fragments.

2.14 Amplification of DNA sequences using the Polymerase Chain Reaction

2.14.1 Preparation of template DNA

1µl of bacterial broth was used to provide the template. Alternatively, a colony was picked from an agar plate with a sterile toothpick and resuspended in 100µl of sterile water; 1µl of this solution was then used in PCR reactions. When purified DNA was used as template it was at a concentration of 50-100ng ml^{-1}.

2.14.2 PCR reaction

A typical PCR reaction mixture consisted of DNA template (1µl), 0.2mM dNTPs, 200pM of each primer (2µl), 5µl of supplier's reaction buffer and 2.5 units of Taq polymerase (Boehringer Mannheim) in a 50 µl reaction. The mixture was briefly vortexed, then centrifuged to collect the sample in the bottom of the tube, and overlayed with mineral oil before cycling. The reaction conditions used in the thermal cycler (Minicycler, MJ Research, Genetic Research Instruments Ltd) were as follows :-

95°C for 5 minutes

Then 30 cycles of :-

95°C for 1 minute

50°C for 1 minute

72°C for 2 minutes

Samples were then analysed by agarose gel electrophoresis on 0.8-2% gels depending on the expected size of the PCR product. If the 50°C annealing temperature resulted in an excess or insufficient PCR products, then the annealing temperature was raised or lowered, repectively.

2.14.3 Design of degenerate primers to homologous regions of *E. coli* arabinose-H+ and Xylose-H+ sugar transporters.

Degenerate primers were designed against the PESPRWL and TVDKA/FGR motifs of the arabinose-H^+ and xylose-H^+ sugar transporters of *E. coli.*

The codons used for the various amino acids are given below. The various codons chosen were based on the frequency with which they are used in the published sequences of genes from *E. coli* (Brown 1991).

	P	E	S	P	R	W	L	
No. of codons	4	2	6	4	6	1	4 =	6912
								Degeneracies
	CCU	GAA	UCU	CCU	CGU	UGG	CUU	
	CCC	GAG	UCC	CCC	CGC		CUA	
	CCA		UCA	CCA	CGA		CUC	
	CCG		UCG	CCG	CGG		CUG	
			AGU		AGA		UUA	
			AGC		AGG		UUG	
Primer 1F	CG	GAA	TCX	CCG	CGX	TGG	CTG	
Primer 2F	CG	GAA	AGX	CCG	CGX	TGG	CTG	
Primer 3F	CG	GAG	TCX	CCG	CGX	TGG	CTG	
Primer 4F	CG	GAG	AGX	CCG	CGX	TGG	CTG	

	T	V	D	K	A/F	G	R	
No. of codons	4	4	2	2	4	4	6 =	6144
								Degeneracies
	ACA	GUA	GAC	AAA	GCC	GGA	CGA	
	ACC	GUC	GAG	AAG	GCG	GGC	CGC	
	ACG	GUG			GCU	GGG	CGG	
	ACU	GUU			GCA	GGU	CGU	
							AGA	
							AGG	
Primer 1R	CG	ACC	GGC	CTT	CTC	AAC	AGT	
Primer 2R	CG	GCC	AGC	TTT	GTC	CAC	AGT	
Primer 3R	CG	ACC	CGC	CTT	CTC	AAC	TGT	
Primer 4R	CG	GCC	TGC	TTT	GTC	CAC	TGT	

X = base inosine

2.15 DNA Hybridisation techniques.

2.15.1 Labelling of DNA Probes.

DNA fragments to be used for probes were labelled with ^{32}P dCTP by random primer extension using a commercially available kit (Multiprime Kit, Amersham Bucks Ltd) according to the manufacturer's instructions. Unincorporated nucleotides were removed by passing the labelled DNA down a G50 Sephadex column (Nick column, Pharmacia).

2.15.2 Southern transfer.

DNA fragments were separated by agarose gel electrophoresis. The gel was photographed and then placed in denaturing solution (0.5M NaOH, 1.5M NaCl) for 45min with continuous agitation (Denley A500 orbital shaker). The gel was then transferred to neutralising solution (0.5M Tris-HCl, pH 7.5, 1.5M NaCl) and left standing for a further 60min. A blotting apparatus was constructed using a Whatman 3MM paper wick, with both ends submerged in 20 x SSC (Maniatis *et al.*, 1982). The neutralised gel was carefully trimmed and placed on the wick. Hybond^{TM} nylon was cut to the size of the gel and soaked in 2 x SSC for 5min. The soaked nylon filter was then placed over the gel making sure all air bubbles had been expelled. One sheet of wet 3MM filter paper (previously soaked in 20 x SSC) was placed over the nylon. 3 sheets of dry Whatman 3MM paper cut to the size of the gel were placed over the wetted paper making sure all air bubbles had been expelled. Approximately 5 sheets of high absorbent blotting paper (Quickdraw, Amersham) were placed on top. The blot was then stacked with several layers of paper towels. Finally a perspex plate and a weight were placed on top of the filters and the apparatus was left overnight. The nylon filter was then removed from the apparatus and lightly dabbed with a tissue to remove excess moisture and each side, then exposed to UV light for 4 min to fix the DNA to the filter.

2.15.3 Colony Blotting.

Where a genomic DNA library could be screened for the presence of a particular gene or fragment of DNA, colony blotting was employed. Colonies to be screened were "tooth-picked" on to media, with a positive and negative control colony, and incubated overnight at 37°C. The plates were dried at 37°C for at least 1hour and subsequently overlayed with a Hybond^{TM} nylon filter, which was pressed down firmly such that when the filter was carefully lifted off the colonies had been transfered to it. The filter was then placed, colony side up, on a Whatman 3MM filter, presoaked with 5% (w/v)

SDS for 5min. The nylon filter was then carefully lifted, dabbed on Whatman 3MM paper to remove excess moisture and then placed on to Whatman 3MM paper pre-soaked with denaturing solution. After 5min the nylon filter was carefully removed, dabbed dry on Whatman 3MM paper and carefully floated, colony side up, on neutralising solution. After 5min the filter was submerged in the neutralising solution and the cellular debris carefully washed from the filter. The filter was then placed on Whatman 3MM paper to remove excess liquid. Finally, the filter was submerged in 6 X SSC solution and then again dabbed dry. The DNA was fixed to the filter by incubation at 80°C for at least 2h.

2.15.4 Hybridisation.

The nylon filter was placed in a glass hybridisation cylinder (Hybaid) and 20ml of prehybridisation solution was added (6 x SSC, 0.1% (w/v) polyvinylpyrrolidone 400, 0.1% (w/v) ficoll, 0.1% (w/v) BSA (fraction V), 0.5% (w/v) SDS and 150µg ml^{-1} denatured calf thymus DNA). It was then incubated in a Hybaid oven set at 65°C for 5h. The labelled probe (section 2.14.1) was denatured by boiling, added to the container and incubated at 65°C overnight. After the hybridisation step the probe solution was removed, and the filter washed first with 300ml of 2 x SSC containing 0.1% (w/v) SDS and then with 0.1 x SSC containing 0.1% (w/v) SDS. Both washes were carried out at 65°C. After washing, the filter was removed, briefly blotted dry on 3MM Whatman paper and wrapped in cling film. An autoradiographic image was recorded on Hyperfilm™-MP (Amersham) which was exposed for 4h with intensifying screens.

2.16 DNA Sequencing.

DNA was sequenced commercially by the dideoxy chain reaction method of Sanger *et al.* (1977) using an ABI PRISM™ dye terminator cycle sequencing ready reaction kit with AmpliTaq ® DNA polymerase, FS. Sequence analysis was performed on the ABI PRISM 377 automated sequencer.

2.17 Preparation of whole cell lysates.

Whole cell extracts of *E. coli* or S-symbionts were prepared by cell disruption via sonication. For *E. coli*, 20ml of mid log phase culture was cooled on ice for 10 min. The culture was then transferred to Oak Ridge centrifuge tubes and harvested by

centrifugation at 7,000 rpm, 4°C for 10 min (Beckman J2-21, JA-20 rotor). The pellet was then washed by resuspension in 10mM phosphate buffer pH 7 or water (for pH profiles) and centrifuged as before. After resuspension in 10ml of 10mM phosphate buffer pH 7 or water, the cells were disrupted using the soniprobe ultrasonicator (Lucas Dawes soniprobe, output settings 8 probe rate 30%) for 1 min on ice. Cell debris were removed by centrifugation at 10,000 rpm, 4°C for 10 min and the lysate was analysed immediately. The same protocol was used to prepare whole cell lysates of S-symbionts; S-symbionts were harvested from 3 blood agar plates after 3-4 days growth at 26°C by scraping the culture off the surface of the agar and suspension in nutrient agar to O.D. 600 of 0.6. 20ml of this S-symbiont suspension was then used to prepare the cell lysates as for *E. coli.*

2.18 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Cell extracts were mixed with an equal volume of 2X sample buffer (Laemmli, 1970) with or without the reducing agent (2-mercaptoethanol) and with or without boiling.

SDS gels were prepared using the Biorad minigel system as per their instructions. The gel solutions were prepared using the recipes given in Maniatis *et al.*, (1982) section 18.52. Either an 8% or 10% separating gel was prepared and poured into the gel formers. The gel was overlayed with propan-2-ol until the gel had set and then it was washed with 1x running buffer (Maniatis *et al.*, 1982). A 4% stacking gel was then poured on top of the separating gel to a depth of about 2cm and gel combs inserted immediately. The gels were then loaded into the running tank and the 2 chambers filled with 1x running buffer. After loading, the gels were run at 25-30mA for about 2 hours or until the tracking dye (bromophenol blue) had migrated to the bottom of the gel.

Once electrophoresis was complete, the gels were removed from their casing and either stained for protein or the proteins were renatured and incubated with chitinase substrates.

2.19 Detection of chitinase activity after SDS-PAGE

Proteins were renatured following SDS-PAGE electrophoresis based on the method of Tronsmo and Harmaan, (1993). Gels were incubated for 20 min in 25% (v/v) aqueous propan-2-ol and then washed 3 times for 30 min with distilled water. Gels were then placed on a clean U.V. Trans-illuminator and covered with a piece of Whatman 3MM paper. The Whatman 3MM paper was soaked with 1ml of 4-methyl-umbelliferyl

chitinase substrates at 20µg.ml^{-1} in 50mM Tris buffer (pH 7). After incubating for 5 min at room temperature, the Trans-illuminator was switched on and the gels were photographed. The position of any band displaying chitinase activity was then marked by puncturing the gel adjacent to the band with a pipette tip. The gels were then stained using either coomassie blue or silver stain. Molecular weight markers (High molecular weight markers, Sigma Chemical Company, Pre-stained Kaleidoscope markers, Biorad Ltd) were used to derive a standard curve of molecular weight against relative mobility (Rf), where the Rf value was determined by dividing the distance each band migrated into the gel by the length of the gel. Rf values of proteins displaying chitinase activity were calculated and their molecular weights determined from the standard curve.

2.20 Visualisation of proteins after SDS-PAGE

2.20.1 Silver staining.

All solutions were prepared as given in the appendix section and stored in the dark. The gel was first removed from the gel apparatus and placed in a tray containing 100ml of fixing solution for 30 min. The fixing solution was then discarded and 100ml of incubating solution added. After 30 min the incubating solution was poured off and the gel was washed 3x for 5 min in 100ml of distilled water. 100ml of silver solution was then added and the gel was incubated for 20 min before being removed, washed briefly with distilled water, and added to 100ml of developing solution. When the protein bands had developed to the required intensity, the gel was placed in stop solution for 10 min, before finally being removed and placed in preserving solution. Gels were then either photographed directly or dried under vacuum onto Whatman 3MM paper (Whatman Labsales Ltd) on a heated gel dryer for 2 hours at 80°C.

2.21 Screening for chitinase producing colonies/cultures.

2.21.1 Screening of gene banks for chitinase producing clones.

Gene banks were screened for clones producing chitinase activity in *E. coli*, a background that does not exhibit chitinase activity. Suspensions of transformants were diluted and plated out so that approximately 500 colonies were visible on each plate after overnight incubation at 37°C. Plates were then placed in the refrigerator for 10 min before being overlayed with a Hybond^{TM} nylon filter; the position of the filter was recorded with reference to marks on both the plate and filter. The filter was

subsequently lifted from the surface of the agar and placed colony side up onto Whatman 3MM paper that had been soaked in 2% (v/v) toluene/ethanol solution. After 5 min incubation the filter was transferred to Whatman 3MM paper soaked in 20µg.ml^{-1} solution of 4-methyl umbelliferyl chitinase substrates in 50mM TRIS buffer, pH 8. The assembly was incubated for 5-20 min until fluorescent halos were seen around some colonies under U.V. illumination (U.V. Transilluminator, U.V. products model TM-20). The positions of putative chitinase producing colonies were marked with a soft lead pencil and the corresponding colony isolated from the master plate.

2.21.2 Screen for detecting chitinase producing cultures

The culture screen is an adaptation of the above colony screen. Five ml of minimal salts agar containing 20µg.ml^{-1} of 4-methyl umbelliferyl chitinase substrates were poured into a mini petri dish (50mm Sterilin) and left to solidify. A number of holes were then punched into the agar with the end of a sterile pasteur pipette to form small wells and each well was used to test putative chitinase producing cultures by adding 50µl of culture, 20µl of 2% (v/v) toluene in ethanol and 30µl of 50mM TRIS buffer pH 8. Incubation at 37°C for 20 min was followed by U.V. transillumination. Positive cultures displayed halos of blue fluorescence surrounding the well.

2.22 Preparation of glycol chitin and regenerated chitin

Glycol chitin and regenerated chitin were prepared from glycol chitosan and chitosan, respectively, by modification of the method of Molano *et al.*, (1977). Essentially, 1g of chitosan or glycol chitosan was ground in a mortar while slowly adding 20ml of 10% (v/v) acetic acid. The mortar was then covered with cling film and allowed to stand overnight at room temperature to allow the chitosan to dissolve completely. 180ml of methanol were added into the mortar and the contents mixed slowly. The resultant cloudy solution was filtered through glass wool in a Buchner funnel. The filtrate was placed in a beaker and stirred with a magnetic stirrer as 3ml of acetic anhydride were added. After approximately 1 min the mixture gelled and the magnet came to a standstill. The gel was allowed to stand for 30 min and then cut up with a spatula. The liquid that oozed out of the gel was poured off and the gel fragments were homogenised at maximum speed for 1 min. The regenerated chitin was finally washed to neutrality and dried; alternatively, the glycol chitin was dried and resuspended in an appropriate volume of water.

2.23 Assay of various chitinase activities using para-nitro-phenol chito-oligosaccharides.

Endochitinase, exochitinase and N-acetylglucosaminidase activities were assayed using the substrates $PNP(GlcNAc)_3$, $PNP(GlcNAc)_2$ and PNPGlcNAc, respectively. Experimental samples (50µl) were assayed in triplicate in micro-Eppendorf tubes. 50µl of a 300µg.ml^{-1} solution of substrate was added and the sample mixture and controls were left to incubate at 26°C for 4-16 hours depending on the velocity of the reaction. The reaction was stopped by the addition of 25µl of 0.4M sodium carbonate. After stopping the reaction the reaction products were centrifuged for 1 min at full speed in a micro-centrifuge. The supernatant was then placed in a well of a microtitre plate and the absorbance was read at 405nm. Control samples consisted of cell lysates boiled for 10 min and samples with no protein. The highest control value was then removed from the appropriate sample readings and the chitinase activity determined using the formula;

$$U = \frac{\Delta A / 1.84 \times 1.74}{T} \quad \mu gPNP.h^{-1}$$

where ΔA = change in absorbance at 405nm

T = time in hours

When activities between different S-symbiont strains were compared, specific chitinase activity was calculated as the amount of PNP generated per hour per mg of protein, one unit being defined as the amount of enzyme required to produce 1µg PNP per hour. The amount of protein in each sample was estimated by Folin Lowry micro-assay (section 2.24).

2.24 Protein estimation

Protein concentrations were estimated using a Sigma Folin-Lowry microassay against BSA standards.

Experimental samples/standards (100µl) of (50µg- 400µg) were placed in microtitre wells in triplicate. To these samples/standards 100µl of Lowry agent were added and the mixture was left to incubate for 20 min at room temp. 50µl of Ciocalteu reagent were then added, mixed and left to incubate for a further 30 min. The assay was then read in a plate reader at 690nm and protein concentrations of samples estimated from the standard curve.

2.25 Determination of heat stability of cloned chitinase.

Whole cell lysates of *E. coli* DH5α containing pMAT3 were prepared as described in section 2.16. 200µl portions in micro-Eppendorf tubes were placed in a thermal cycler (Minicyler, MJ Research, Genetic Research Instruments Ltd, Dunmow, Essex), and heated from 30^0C - 45^0C at 5^0C intervals for ten minutes each, and then from 48^0C - 54^0C at 1^0C intervals for ten minutes. At each time interval an micro-Eppendorf tube containing 200µl of sample was removed from the thermal cycler and chitinase activity was assayed in triplicate using PNP$(GlcNAc)_2$. Assays consisted of 50µl of heat-treated sample and 50µl of a 300µg.ml^{-1} solution of PNP$(GlcNAc)_2$ in 10mM phosphate buffer, pH 7. The assay was left at 37°C for 2 hours and read at 405nm, after the addition of 25µl 0.4M sodium carbonate. The percentage enzyme activity that remained after each incubation was determined.

2.26 Growth of S-symbiont in nutrient agar chitin/glycol chitin

The S-symbiont isolated from *G. m. morsitans* was grown on blood agar at 26°C for 3-4 days under reduced oxygen conditions and then harvested by scraping cells from the surface and suspending them in nutrient broth. The S-symbiont suspension was then adjusted to give an O.D. 600 of 0.6 and 100µl portions used to inoculate three universals containing 10ml of nutrient broth and three universals containing 10mls of nutrient broth containing 1% regenerated chitn and 1% glycol chitin. These cultures were then incubated at 26°C for 4 days under reduced oxygen conditions. The cells were subsequently adjusted to O.D 600nm of 0.6 and 800µl of each culture was used to prepare whole cell lysates (2.17). 50µl of each cell lysate was then assayed in triplicate (2.23) and the specific activities determined.

2.27 Effect of pH on the activity of S-symbiont chitinase.

Whole cell lysates of *E. coli* containing pMAT3 were assayed for chitinase activity using PNP$(GlcNAc)_2$ over a range of pH values from 3-11 using appropriate buffers. Assays were essentially identical to the one described in 2.25, except that the pH was varied.

2.28 Screening of gene banks for N-acetylglucosamine transporter gene.

Electrocompetent *E. coli* LR2-167 prepared as described (section 2.9.2) were transformed (section 2.9.4) with S-symbiont gene banks, prepared as described (section 5.2.1). Cells were left to grow for 30 minutes in SOC medium containing N-acetylglucosamine, before being washed in minimal salt medium (2.6.3). The cells were then plated on minimal agar containing N-acetylglucosamine and ampicillin (section 2.6.7).

Section 3

Culture and transformation of the secondary endosymbiont of Glossina morsitans morsitans

3.1 Introduction

Bacterial endosymbionts are found in at least 10% of insect species but knowledge about them is tempered by the failure to cultivate them axenically, either in cell culture or in other insects. Consequently classification attempts have been quite limited and at present only three monospecific genera of insect symbionts are listed (Bergey, 1984; Bergey, 1994).

Knowledge about insect symbionts is based mainly on five lines of investigation;

1. Examination of insects to determine the location/distribution of the symbiont in the various insect stages, leading to a knowledge of the degree of adaption to their host and mechanisms of their hereditary transmission.
2. Characterisation by staining methods and light microscopy of the symbionts.
3. Elucidation of the physiological role of the symbiont is determined, where possible, by removal of the symbiont by use of antibiotics, lysozyme or elevated temperature to produce aposymbiotic insects (free from symbionts). The effects of the removal of the symbiont on the insect are noted; any nutrient deficiencies which arise are tested by diet supplementation with vitamins and nutrients. Ideally the symbionts are cultured and then re-introduced to satisfy Koch's postulates.
4. Isolation of the symbiont and where possible their biochemical characterisation and analysis of DNA base ratios.
5. More recently 16SrRNA sequence analysis has been performed to determine where they should be placed phylogenetically (Beard *et al.*, 1992; Aksoy, 1995a; Aksoy, 1995b; Aksoy *et al.*, 1995)

The tsetse fly has been shown to harbour at least three different bacteria (O'Neill *et al.*, 1993); Primary endosymbionts (P-endosymbionts), secondary endosymbionts (S-symbionts), previously named rickettsia-like organisms, and *Wolbachia* (bacteria associated with cytoplasmic incompatibility, see section 1.1.5). The P-endosymbionts were initially identified in the early part of the twentieth century (Stuhlman, 1907; Roubaud, 1919, Wigglesworth, 1929). These large P-endosymbionts (3-9μm in length and 1-1.8μm in width) are harboured inside specialised cells (mycetocytes) forming a white U-shaped organelle called the mycetome. Similar bacteria have been found in a wide variety of insects. The smaller organisms (S-symbionts) measure 0.5μm by 2μm and have been described in tissues of the ovaries, midgut epithelium, in the surrounding

musculature, in cells associated with the fat body and haemolymph, as well as the midgut mycetome (Reinhart *et al.*, 1972; Pinnock and Hess, 1974; Heubner and Davey, 1974; Welburn, 1991). Pell and Southern (1975) outlined the differences between P-endosymbionts and these smaller bacteria. The S-symbionts demonstrated features characteristic of rickettsia including the presence of a lytic zone and a host cell membrane surrounding the organism whereas P-endosymbionts were not surrounded by host cell membrane and had structures that are typical of Gram negative bacteria. The P-endosymbiont of *Glossina* has been maintained viable for 85 days in a mycoplasma medium supplemented with pyruvate, succinate and a combination of nucleotides (Wink, 1979). Insects have also been rendered aposymbiotic for the P-endosymbiont using a number of different techniques including antibiotic treatment, lysozyme treatment, feeding on rabbits immunised against purified symbionts and feeding on rabbits treated with antibiotics (Jordan and Trewern, 1973 and 1976; Pell and Southern, 1976; Nogge, 1976; Schlein, 1977). These treatments led to a loss of fecundity and prolonged treatment resulted in degeneration of the ovaries, believed to be due to the loss of vitamins normally provided by the endosymbionts. The vitamin deficiency can be overcome by supplementation of the diet of aposymbiotic flies with B vitamins, and the production of B vitamins by endosymbionts has been demonstrated by microbiological assays (Nogge, 1978 and 1980).

Recently, the 16SrRNAs of P-endosymbionts isolated from five species of tsetse (representing the three major sub-genera of the genus *Glossina*) were sequenced. Analysis of these sequences indicated that the P-endosymbionts constitute a distinct lineage related to the family Enterobacteriaceae, within the gamma-3 subdivision of the Proteobacteria. The phylogeny appears to parallel the classic taxonomic assignments independently developed for their insect host species, which is suggestive of an ancient association between the fly and the symbiont (Aksoy *et al*, 1995). However, differences in signature sequences and genomic organisation distinguish the P-endosymbiont from other members of the family Enterobacteriaceae and also from the genus *Buchnera* (aphid symbionts related to P-endosymbionts) suggesting that these symbionts should be classified in a new genus (Aksoy, 1995a). The genus *Wigglesworthia*, and a new species *Wigglesworthia glossinidia* have been proposed the P-endosymbiont found in the mycetocytes of *Glossina morsitans morsitans* being designated as the type strain of this species.

The basis of the symbiosis of the S-symbiont with its insect host is thought to be enhanced puparial survival for insects harbouring these organisms (Baker *et al.*, 1990; Maudlin, 1991). Welburn et al., (1987) isolated and cultured these organisms *in vitro* (in *Aedes* cell culture) from haemocytes of seven species and sub-species of tsetse (Welburn *et al* 1987). The S-symbionts were pleomorphic, consisting of electron dense small round variants and large elongated less electron dense variants. They classified these bacteria as members of the genus *Wolbachia*, of the order *Rickettsiales*, on the basis of morphology, intracellular association with insect cells and pathological studies. This classification was subsequently challenged by Beard *et al.* (1993b) who isolated the same bacterial endosymbionts from *Glossina morsitans morsitans* and *Glossina pallidipes* and subjected them to 16SrRNA sequence analysis and phylogenetic analysis. The 16SrRNA sequence analysis placed them in the gamma subdivision of the Proteobacteria which are phylogenetically distinct from most members of the *Rickettsiaceae*, which align with the alpha subdivision. Although the S-symbionts are placed within the gamma subdivision they do not appear to be closely related to any organism of the group for which the 16SrRNA is known (figures 3.1, 3.2) (Beard *et al.*, 1993b). Comparison of the two different isolates revealed that they were very closely related. The base sequence was identical over the 1,117 bp region of 16srRNA amplified, indicating that these isolates belong to the same genus. These symbionts therefore bear no relationship to rickettsia-like organisms capable of invading eukaryotic cells and will be called, in this thesis, the secondary symbionts of tsetse.

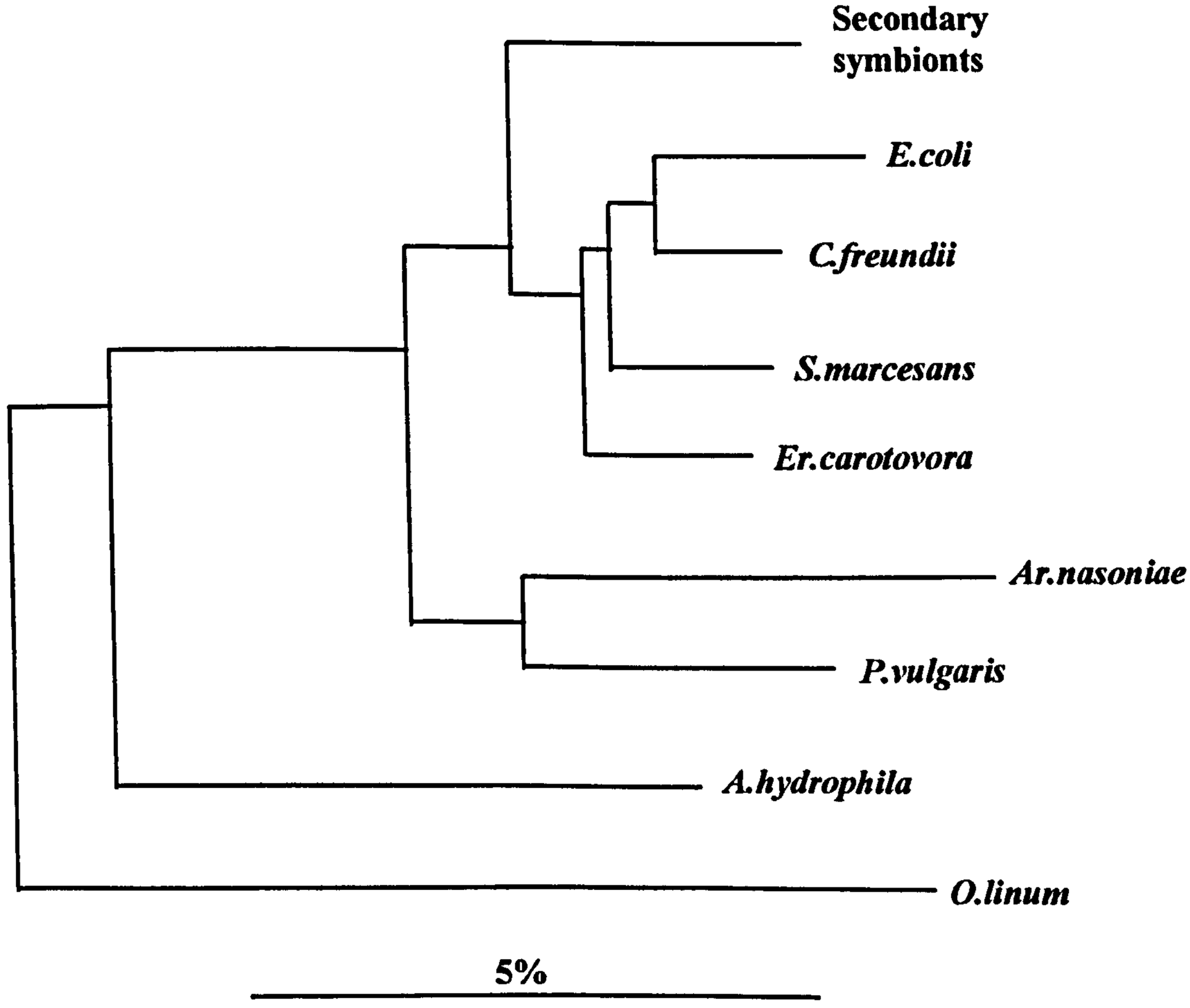
Beard *et al.*, (1993b) also grew the endosymbionts in cell free media although they record that attempts to grow these bacteria on various types of agar plates under aerobic, anaerobic or microaerophillic conditions failed.

This section describes the development of pure culture techniques, classical biochemical tests and transformation of the S-symbiont of the tsetse fly.

Figure 3.1 Evolutionary distances among 16SrRNA of various enteric bacteria and their relatives (taken from Beard *et al.*, 1993b)

Species	Evolutionary distance							
1. Endosymbiont morsitans	-							
2. *Escherichia coli*	5.7	-						
3. *Citrobacter freundii*	4.8	3.0	-					
4. *Serratia marcescens*	4.9	4.0	2.8	-				
5. *Erwinia carotovara*	4.6	4.8	2.8	2.9	-			
6. *Pseudomonas vulgaris*	7.5	7.3	7.5	6.5	6.7	-		
7. *Arsenophonus nasoniae*	8.0	9.1	8.6	8.3	7.9	6.8	-	
8. *Aeromonas hydrophila*	11.3	10.9	11.6	11.2	10.6	11.4	12.9	-
9. *Oceanus linum*	14.9	15.9	14.6	15.0	14.8	15.4	16.6	14.1
Species	1	2	3	4	5	6	7	8

Figure 3.2 Dendogram displaying phylogenetic relationships of tsetse secondary symbiont and other Gram negative bacteria (taken from Beard *et al.*, 1993b)



3.2 Results

3.2.1 Initial growth experiments

Initial experiments performed involved plating out the S-symbiont on nutrient agar. S-symbionts were recovered from *Aedes albopictus*/S-symbiont culture (Welburn *et al.*, 1987) (figure 3.3) and plated out at high cell density on nutrient agar. Growth was initially observed after incubation for 1 week at 26°C, but only in cracks that had been produced by accident with the loop used to spread the bacteria on the plate. The bacteria that were growing in these cracks were stained using the Gimenez technique (Gimenez, 1964) and appeared identical to the bacteria originally isolated from the fly (see figure 3.3). The bacteria were subsequently grown in liquid culture (nutrient broth). Plasmids from these cells were isolated and analysed by agarose gel electrophoresis (see figure 3.7 lanes 2 and 4) when compared with a plasmid preparation from the original bacteria. The results revealed an identical plasmid profile, indicating that the bacteria were the same as those originally isolated from the tsetse fly. Attempts to grow this organism under anaerobic conditions failed. This finding together with the observation that these bacteria grew in cracks in the agar rather than on the surface prompted the conclusion that the bacteria might be microaerophilic.

3.2.2 Growth of S-symbiont in agar deeps (This experiment was performed by Mr C.Dale and is included in his PhD thesis)

S-symbionts were recovered from *Aedes albopictus* / S-symbiont culture, as described in section 2.9.3. The pellet containing the S-symbionts was resuspended in 1ml of nutrient broth and used to inoculate a number of agar deeps containing 0.4% agar in nutrient broth. Following incubation for five days at 26°C, growth of the S-symbiont was observed as a thin film just below the surface of the agar deep (figure 3.4). Following this finding, an agar plate culture method was developed.

3.2.3 Growth of S-symbiont on blood agar plates

The S-symbionts were grown on blood agar incubated under reduced oxygen conditions produced by flushing an anaerobic gas jar with a gas mixture of 5% oxygen 85% nitrogen and 10% carbon dioxide containing the sheep-blood agar plates (section 2.2) inoculated with *Aedes albopictus*/ S-symbiont culture. The gas jar was sealed and incubated for four days at 26°C, after which a lawn of bacterial growth was observed.

These bacteria were then sub-cultured on fresh blood agar (figure 3.5). From this culture a single colony was taken and streaked on to blood agar and the plate was incubated at 26°C under reduced oxygen as before. This process was repeated twice before extrachromosomal replicating elements (ERE) were extracted from the pure culture. These where shown to have an identical ERE profile to that from the symbionts originally isolated from the fly. This finding confirmed that the S-symbionts can be grown, in pure culture, outside of insect cells. Subsequently it was shown that the symbionts also grow on nutrient agar incubated under reduced oxygen conditions, but growth obtained was much slower and biomass more sparse than on blood agar. The S-symbionts were Gram stained and found to be Gram-negative (figure 3.6)

Figure 3.3 Growth of S-symbiont in *Aedes albopictus* insect cell culture.
stained Gimenez, (1964). Photograph courtesy of Dr Ian Maudlin

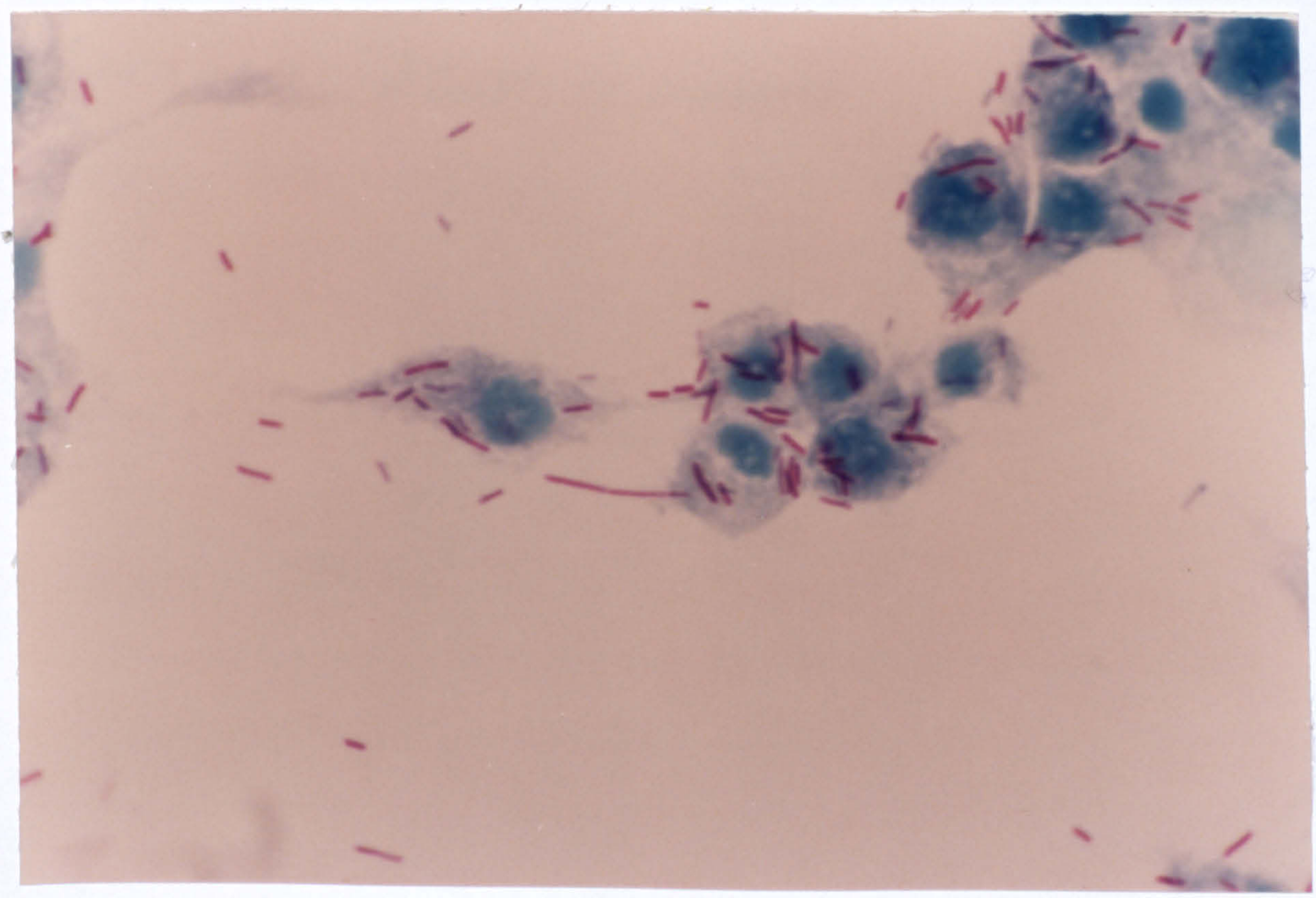


Figure 3..4 Growth of S-symbiont in agar deeps. (photograph courtesy of C.Dale)

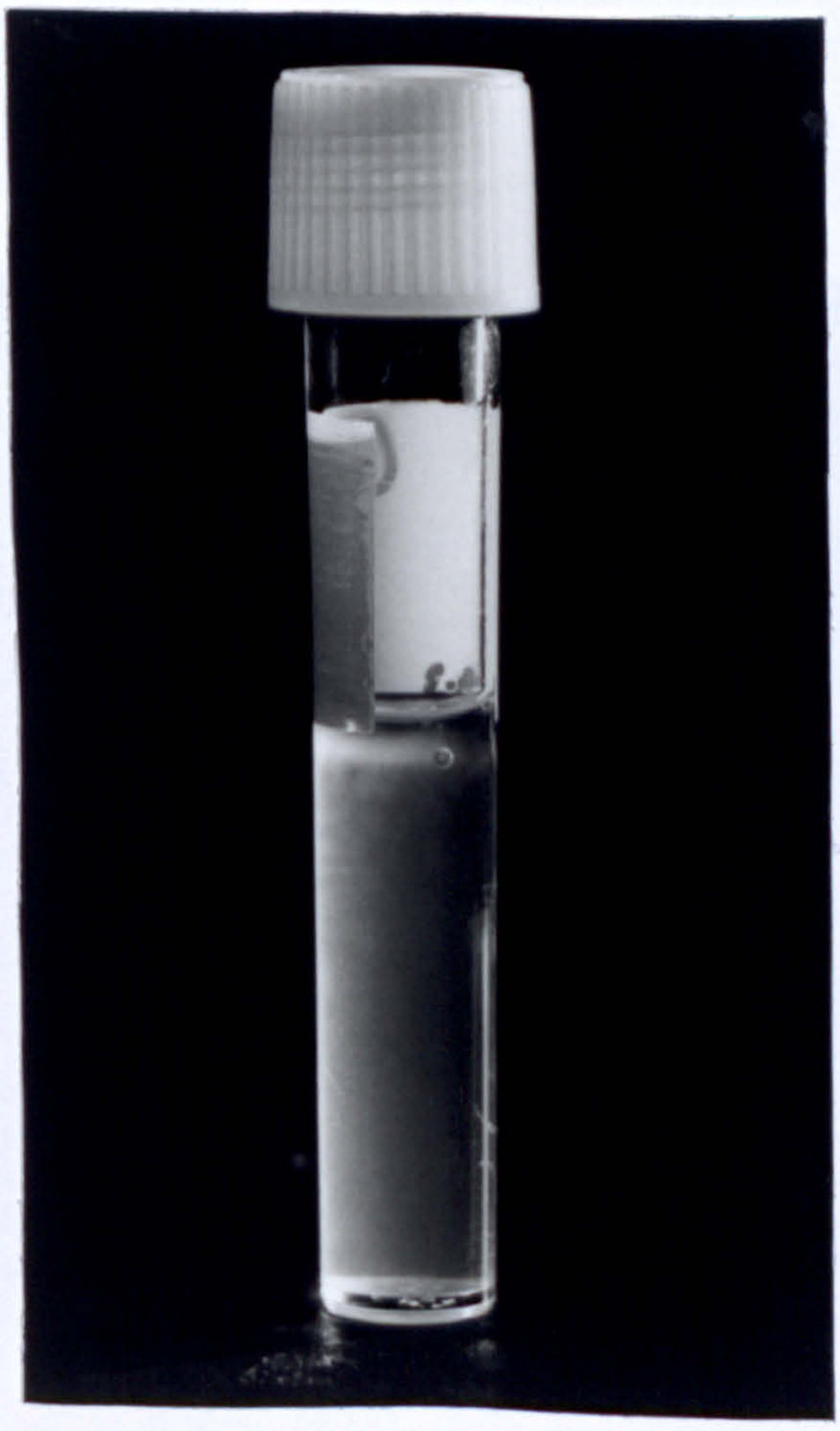


Figure 3.5 Growth of secondary symbionts on blood agar plates.

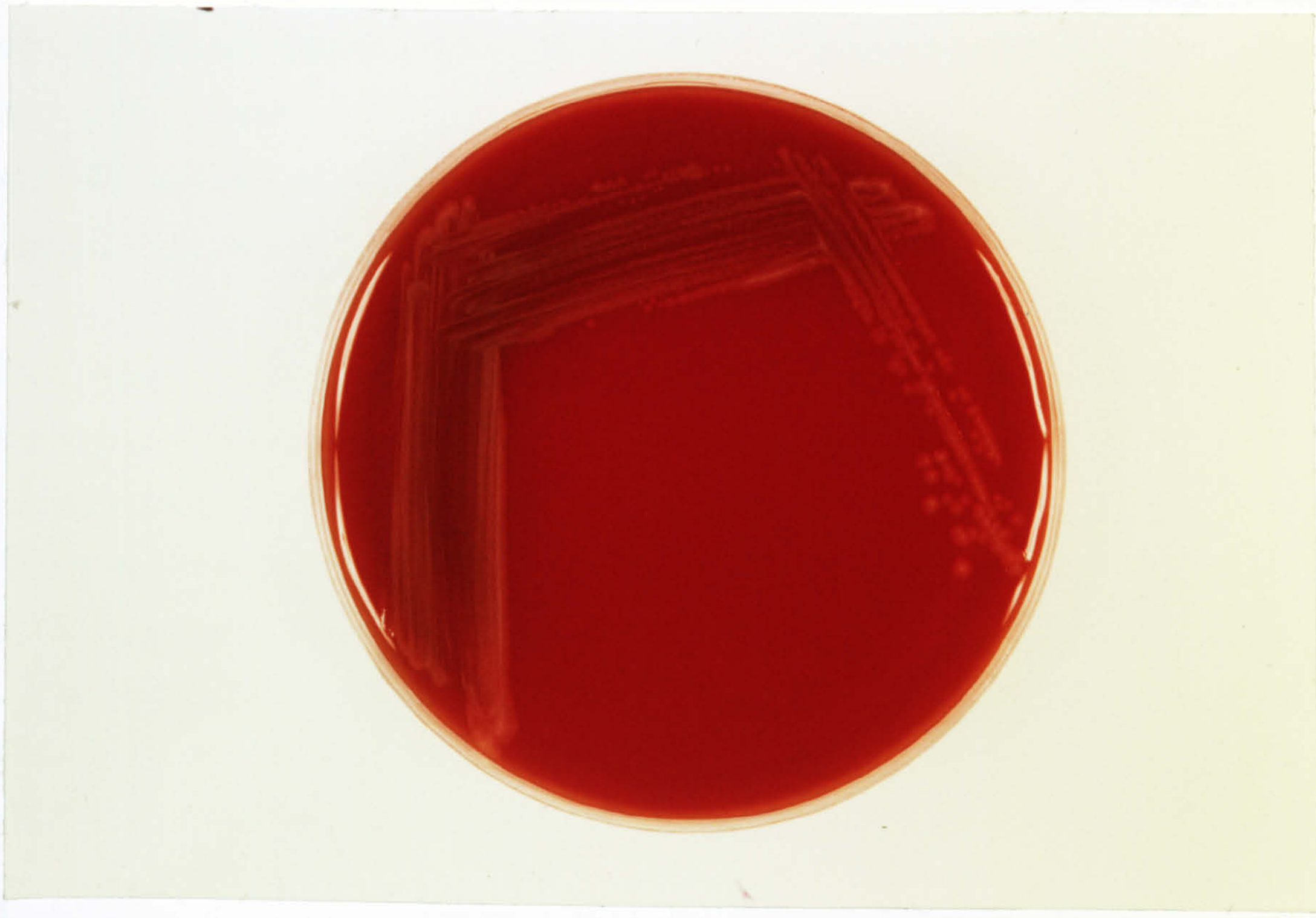
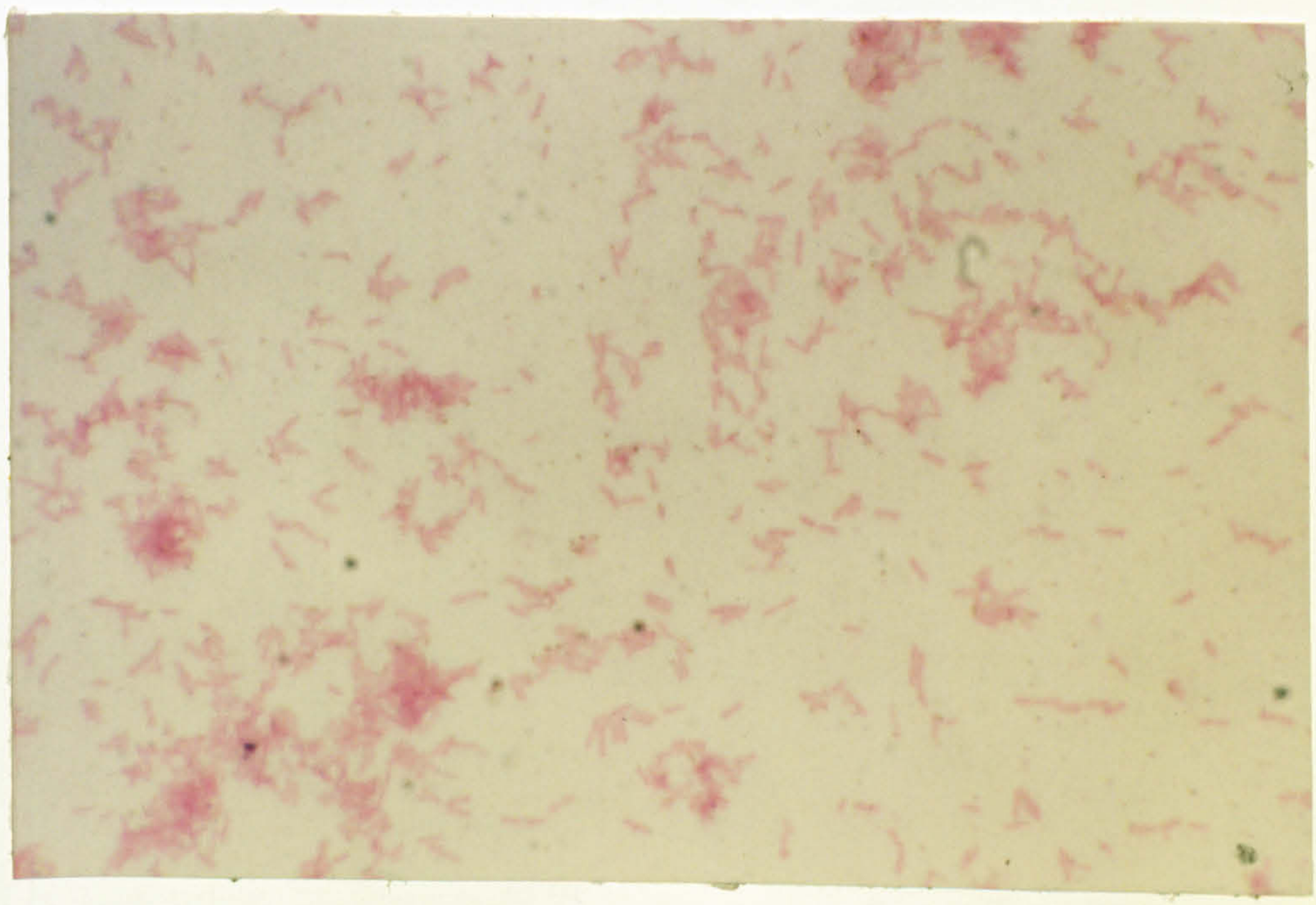


Figure 3.6 Gram stain of secondary symbionts



3.2.4 Testing for contamination and identification of S-symbiont

Cultures of the S-symbiont were routinely tested for contamination throughout the course of the work described in this thesis. The symbiont cannot grow at temperatures above 28°C; therefore, cultures suspected of being infected were streaked onto blood agar and the plates were incubated at 37°C. Culture growth under these conditions indicated the presence of contamination.

Three approaches were also taken to ensure that the S-symbionts used throughout this thesis were indistinguishable from the S-symbionts studied in other research establishments; Firstly the restriction profile of S-symbiont ERE DNA was used to identify the S-symbiont (figure 3.7). Secondly specific PCR primers designed from one of the extrachromosomal replicating elements of the S-symbiont by a group working in Yale university USA, were used to amplify a 1.2kb product which is diagnostic of the S-symbiont (O'Neill *et al.*, 1993; figure 3.7 Lanes 6-8). Finally, a *Pst*1/*Hin*dIII 1.8 kb fragment of S-symbiont plasmid DNA, cloned within pBluescript SK^+ and coding for the enzyme DNA helicase I was used as a probe to ensure that the bacterium studied in this thesis was indistinguishable from the bacterium the tsetse group in Glasgow were studying (figure 3.8). This plasmid was a kind gift from Mr C. Dale. (Division of molecular genetics, University of Glasgow.

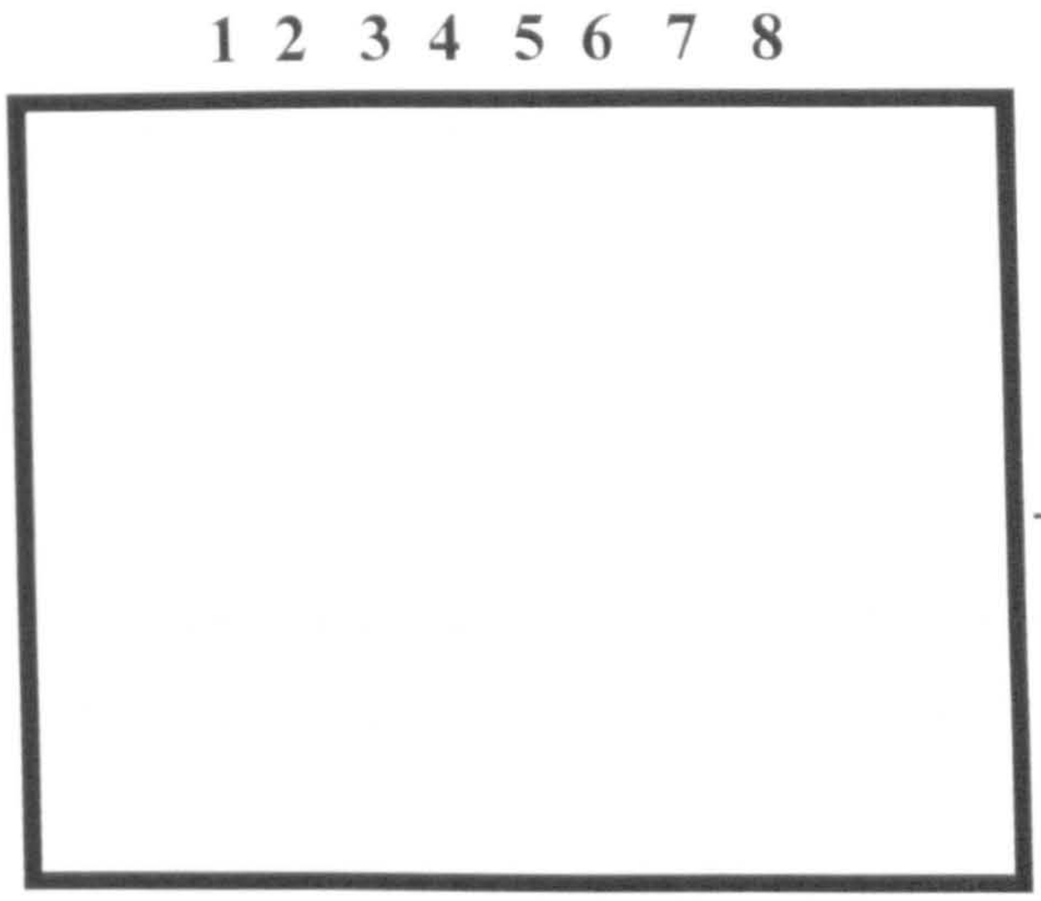


Figure 3.7 Visualisation of plasmids in the S-symbiont and its transformant.
Lane 1, plasmids isolated from S-symbiont (pGem3ZKm) digested with *Eco*R1. Lane 2, plasmids isolated from S-symbiont digested with *Eco*R1. Lane 3, pGem3ZKm digested with *Eco*R1. Lane 4, S-symbiont plasmids uncut. Lane 5, 1kb DNA molecular size marker. Lanes 6-8, PCR products using primers specific to plasmid from S-symbiont (*G. m. morsitans*) and template DNA from the S-symbiont isolated from *G. m. morsitans*, *G. p. palpalis* and *G. austeni*, respectively.

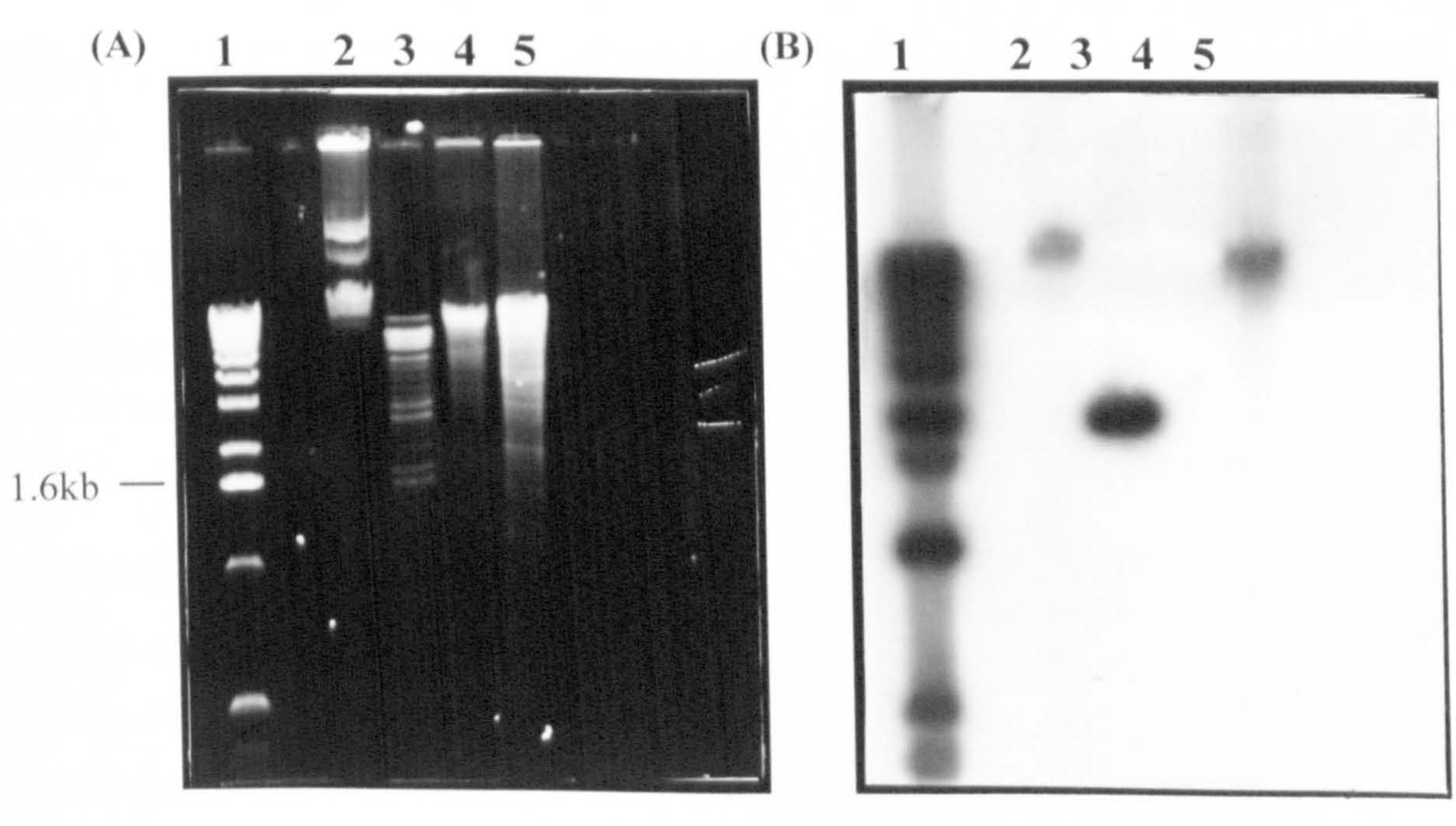


Figure 3.8 Hybridisation of S-symbiont probe to S-symbiont plasmid sequences.
S-symbiont genomic DNA prepared from single colonies isolated from blood agar plates was probed with a fragment of extrachromosomal replicating DNA isolated from S-symbiont (*G. m. morsitans*). (A) Lane 1, 1kb DNA molecular size marker. Lane 2, S-symbiont extrachromosomal replicating elements. Lane 3, ERE restricted with *Pst*I *Hin*dIII. Lane 4, *E. coli* genomic DNA cut with *Eco*R1. Lane 5, S-symbiont genomic DNA cut with *Eco*R1.
(B) Southern blot of gel (A) probed with *Pst*I *Hin*dIII fragment of S-symbiont (*G. m. morsitans*) ERE DNA.

3.2.5 Transformation of tsetse secondary symbionts

Attempts to transform the S-symbionts were made with three different plasmids. These were pUC18, pGem3Z containing the kanamycin gene from Tn903 cloned into the *Eco*R1 site (pGem-3zKm) and plasmid pYZ291 containing the *Serratia marcescens* chitinase gene. A range of different temperatures between 25-42°C were used for the heat shock (table 3.1)

Temperature of heat shock and tube number

Plasmid used for transformation	42°C	40°C	38°C	36°C	34°C	30°C	25°C	Antibiotic selection
pYZ 291	1	2	3	4	5	6	7	Kanamycin
	8	9	10	11	12	13	14	25µg.ml^{-1}
pGem 3-z	15	16	17	18	19	20	21	Kanamycin
	22	23	24	25	26	27	28	25µg.ml^{-1}
pUC18	29	30	31	32	33	34	35	Ampicillin
	36	37	38	39	40	41	42	20µg.ml^{-1}
Control (no plasmid)	43	44	45	46	47	48	49	No selection

Table 3.1 Transformation of S-symbiont.
49 separate tubes of competent S-symbiont (section 2.8.1) were subjected to transformation using a range of different heat shock temperatures and plasmids the cultures were incubated at 26°C for 12 hours before antibiotic selection pressure was applied.

Of all the tubes of competent S-symbiont used in the transformation experiment (table 3.1), growth was observed only in tubes 5 and 22. Of these, only the culture in tube 22 (S-symbiont transformed with the plasmid pGem3ZKm) proved to be the S-symbiont, as judged by the plasmid profile (figure 3.7); tube 5 contained a contaminant.

3.2.6 Stability of transformed S-symbiont

The transformed S-symbiont (section 3.2.3) was maintained in nutrient broth at 26°C for four weeks without antibiotic selection. The S-symbiont being subcultured every 3-4 days. After this time period the S-symbiont was subjected to selection with kanamycin at 25 µg.ml^{-1} and was shown to have retained kanamycin resistance, whereas

the controls were susceptible. Plasmid preparations were subsequently extracted from the transformed bacteria and digested with *Eco*R1, producing the plasmid profile expected from digestion of the S-symbiont plasmids with 2 additional bands corresponding to the plasmid pGem 3ZKm (figure 3.7). The results therefore suggest that this plasmid was stabily maintained over 28 days without antibiotic selection.

3.2.7 Classical biochemical tests for the classification of the S-symbiont of *G. m. morsitans*

Once pure cultures of the S-symbiont had been obtained it was possible to perform a number of biochemical tests on the bacterium to further characterise the organism.

3.2.7.1 Acid production by S-symbiont grown in different sugars.

Cultures of S-symbiont originally isolated from *Glossina m. morsitans* were tested for the ability to ferment various sugars. Acid production (colour change from red to yellow) was scored by eye against a control culture containing no sugar (table 3.2).

Sugar	24h	48h	72h
Glucose	-	+	++
Galactose	-	-	-
N-Acetylglucosamine	-	++	+++
Adonitol	-	-	-
Arabinose (L)	-	-	-
Dulcitol	-	-	-
Inulin	-	-	-
Lactose	-	-	-
Mannitol	-	+	++
Mannose	-	-	-
Raffinose	-	-	-
L-Rhamnose	-	-	-
Salicin	-	-	-
Sucrose	-	-	-
Trehalose	-	-	-
Xylose	-	-	-
D-glucosamine	-	-	-
Fructose	-	-	-
Maltose	-	-	-
Starch	-	-	-
Ribose	-	-	-
Cellobiose	-	-	-
Glycerol	-	-	-
Myo-inositol	-	-	-
Melibiose	-	-	-
Sorbose	-	-	-
fructose	-	-	-

Table 3.2 Growth of S-symbiont in various sugar broths

- no colour change

+ slight colour change (red-orange/red)

++ colour change (red-yellow/orange)

+++ deep colour change (red-bright yellow)

3.2.7.2 classical biochemical tests

Biochemical tests were performed as described in section 2.3. The results are noted in Table 3.3

Table 3.3 Results of biochemical tests

Catalase	Negative
Oxidase	Negative
Gelatin liquefaction	Negative
Gram stain	Negative (figure 3.2)
Morphology	Pleomorphic rods and cocci (figure 3.2)
Motility	Non-motile
Hydrogen sulphide production	Negative
Oxygen requirement	Yes- Microaerophillic (figure 3.1)
Urease	Negative
Nitrate reduction	Negative
Growth in the presence of bile salts	Negative
β-galactosidase	Negative
Voges-Proskauer Test (butanediol fermentation pathway)	Negative
Indole production	Negative

3.4 Discussion

The S-symbiont of *Glossina m. morsitans* was cultured (without insect cells) in liquid culture, where it grew to a maximum O.D. at 600nm of approximately 0.35 after 3 to 4 days of growth at 26°C, as well as on blood agar plates under reduced oxygen conditions. The S-symbiont also grew in agar deeps forming a ring of biomass just below the agar surface which indicates that it is microaerophillic. The relatively low level of growth in liquid culture may be associated with the build up of toxic oxygen intermediates inhibiting growth beyond this cell density. In contrast, the rich growth on blood agar is possibly due to the presence of catalase in this medium which compliments the microaerophillic nature of the S-symbiont. However, it was found that incubation on blood agar under normal atmospheric conditions promoted very slow and sparse growth, and this only when a large inoculum was applied, suggesting that the presence of catalase, as well as reduced oxygen conditions, may be needed for optimum growth. Beard *et al.*, (1993b) also attempted growing *Glossina m. morsitans* under microaerophilic conditions but without success, presumably because they did not use the combination of blood agar and reduced oxygen conditions used in the studies presented here. That this symbiont is closely related to the one used by Beard *et al.*, (1993b) and O'Neill *et al.*, (1993) was confirmed by the fact that PCR primers designed to amplify a 1.2kb segment of one of the ERE's of the S-symbiont by them, also amplified a fragment of identical size from the bacteria grown on blood agar. Additionally, the profile of fragments generated by digestion of S-symbiont extrachromosomal elements with *Eco*R1 was identical to those generated by similar digestion of S-symbiont ERE's (isolated from *Glossina m. morsitans*) by Beard *et al.*, (1993b). That the symbiont cultured on blood agar is closely related to the bacterium that Mr C Dale was investigating is also borne out by the fact that a probe produced from a fragment of one of the ERE's of S-symbiont (isolated from *G. m. morsitans*) by him hybridises with a fragment of identical size, from the plasmids isolated from the isolate of symbiont used in this thesis. This all corroborates the contention that the isolate characterised here is indistinguishable from isolates of the S-symbiont studied in these other laboratories and therefore must be the same species of bacterium.

The ability to produce pure cultures of this organism opens up a number of lines of study and has been invaluable in this study because it has allowed the production of large amounts of the S-symbiont in order to produce genomic DNA for molecular studies. This was both expensive and unreliable when culture relied on growth in insect cell lines (Welburn *et al.*, 1987). The S-symbiont was routinely grown on blood agar under reduced oxygen conditions and was maintained for eighteen months in pure culture during the later part of this study. Growth on agar also makes the molecular study of organisms more amenable; single colonies are easily recovered, in particular the production of cultures from isolated single colonies has established unequivocally the fact that large extra-chromosomal replicating elements are carried by each individual of the bacterial population and not as a heterogeneous mix of different elements carried by different individuals.

All attempts to cure the S-symbiont of these large self replicating extra-chromosomal elements has been unsuccessful (Mr C Dale, Division of Molecular Genetics, Glasgow University, personal communication). They may possibly be mini chromosomes and therefore, the reference to such elements as plasmids in the literature (Beard *et al.*, 1993b) is unmerited. Furthermore, the presence of similar elements with very similar restriction profiles in all S-symbiont strains isolated from different tsetse species (Beard *et al.*, 1993b; O'Neill *et al.*, 1993; Toleman,1993) suggests that they encode proteins which are vital to the existence of these symbionts in their particular environmental niches. That these elements are very similar is further illustrated by the fact that PCR primers designed to amplify a 1.2kb fragment of DNA of one element from the S-symbiont isolated from *Glossina pallidipes*, also amplify an identically sized fragment from all strains of tsetse tested (O'Neill *et al.*, 1993).

Only one attempt at transforming *G.m.morsitans* was successful; plasmid pGem 3Z containing the kanamycin gene from Tn903 was successfully used to transform the S-symbiont using the calcium chloride method. All attempts to transform this bacterium via electroporation were unsuccessful. The pGEM3Z plasmid contains the origin of replication from colE1 and demonstrates the feasibility of transformation. However, the transformation efficiency was very low in that only one of 49 attempts using different protocols (table 3.1) produced transformants. Beard *et al.*, (1993) also transformed this bacterium via a similar method with the broad host range plasmid pSUP204, which is a

RSF1010 derivative belonging to IncQ incompatibility group. Unfortunately no indication of the ease of its transformation was provided, although the authors do state that repeated attempts at transforming the similar symbiont isolated from *G. pallidipes* were unsuccessful, which they suggest could be due to incompatibility between the endogenous "plasmids" and the RSF 1010 based vector. This explanation is, however, considered to be highly unlikely, since the same paper records that the plasmids isolated from the S-symbiont (*G. m. morsitans*) have virtually identical restriction profile to the plasmids isolated from the S-symbiont (*G. pallidipes*) and therefore would almost certainly belong to identical incompatibility groups. It is more likely that the transformation of the S-symbionts of tsetse is a rare event in general, with the transformation protocols tested, which would also explain why it proved impossible to isolate chitinase mutants from this bacterium via allelic exchange using the cloned mutated chitinase gene (chapter 5). Mr Colin Dale (Division of Molecular Genetics, Glasgow University) has also had considerable difficulty in transforming this bacterium (personal communication). During attempts to transform this bacterium by the calcium chloride method, plaques were seen on the control plates, which had been processed in a mock transformation and had been plated out without antibiotic selection on blood agar (data not shown). This finding is suggestive of the presence of a phage in this organism, which may be temperate since the plaques were observed following heat shock. However other manipulations involved in this procedure cannot be ignored and subsequent attempts to repeat this experiment were unsuccessful. Virus-like particles have been identified within the ultrastructure of the S-symbiont isolated from *G. m. morsitans* by electron microscopy (Welburn, 1991). The phage responsible for these findings has recently been isolated by Mr C Dale (Division of Molecular Genetics, Glasgow University) and it has been found that it contains DNA coding for a restriction/methylation modification system. The presence of the phage in the S-symbiont would be expected, therefore, to lower the frequency of transformation; accordingly carriage of the phage is a likely reason that the S-symbiont is difficult to transform, since much of the DNA would be digested as it enters the bacterium.

The transformation of the bacterium by the pGEM3ZKm plasmid was demonstrated to be stable in that it was maintained for 4 weeks without antibiotic selection, as judged by restriction digestion of plasmid DNA, as well as continuing to be resistant to kanamycin. The transformation and stable inheritance of plasmid DNA have also been demonstrated by Beard *et al.*, (1993b) and is a major step forward towards the production of pseudotransgenic tsetse.

Biochemical tests performed on pure cultures of the S-symbiont (*G m. morsitans*) revealed that it was catalase negative, which is different to the majority of the Enterobacteriaceae. Its microaerophilic nature explains why it is unable to reduce nitrate, since, this requires anaerobic conditions and the ability is restricted to anaerobic and facultative anaerobic bacteria (MacFaddin 1976) This result is also different from the majority of bacteria belonging to the Enterobacteriaceae, which are generally able to reduce nitrate to nitrite. However, it is not the sole exception since some strains of *Enterobacter agglomerans* and *Yersinia enterocolitica* also are unable to reduce nitrate to nitrite and are classified as belonging to the Enterobacteriaceae. The S-symbiont was unable to grow on bile agar under reduced oxygen conditions and is non-motile. The bacterium did however ferment glucose and mannitol with the production of acid, although not as vigorously as it fermented N-Acetylglucosamine. This is probably associated with the niche which the bacterium normally occupies; the chitinase system of the bacterium would produce N-acetylglucosamine from the breakdown of chitin, whereas glucose would probably be in short supply in the tsetse fly, since its main source of energy is from proline catabolism. The cloned DNA from the chromosome of *G. m. morsitans* gave a G:C ratio of approximately 50%, which is in agreement with the symbiont being placed within the Enterobacteriaceae. Additionally, the successful establishment in the S-symbiont of the colE1 plasmid pGEM3ZKm is also consistent with classification of the bacterium as a member of the Enterobacteriaceae

The niche that the bacterium occupies normally could also explain why it is catalase negative. Presumably within the fly there is a reduced oxygen environment and within insect cells the bacterium would not require detoxifying enzymes such as catalase. In this type of environment it is not difficult to envisage the loss of a redundant enzyme by the bacterium. Overall, therefore the biochemical tests do not disagree with the placement of this organism within the Enterobacteriaceae but rather are consistent with

the phylogenetic placement within this group by on the basis of 16SrRNA. Beard *et al.*, (1993b) suggest that although being members of this group they do not appear to be specifically related to any member within it.

Section 4

Characterisation of the chitinase system of S-symbionts of

Glossina spp.

4.1 Introduction

Section three described the development of a culture system and details of the specific biochemical profile of the S-symbiont of *Glossina m. morsitans*. This chapter deals with the chitinase system of this bacterium and the use of various techniques to characterise this system. The available tests for assessing chitinase activity are reviewed.

A wide variety of methods are used to detect and assay chitinase activity. These assays fall into two major categories, consisting of; those using macromolecular chitin or its derivatives and those using soluble oligosaccharide derivatives (Gooday, 1994). The former category includes: zones of clearing around colonies growing on chitin containing agar media. Zones of clearing around protein bands in gels containing chitin, or around overlay gels containing chitin (Trudel and Asselin, 1989); measurement of the release of reducing sugars from chitin (Ulhoa and Peberdy, 1991; Vasseur *et al.*, 1990) ; measurement of turbidity or viscosity of chitin containing solutions (Reyes *et al.*, 1989; Lundblad *et al.*, 1974 and Jeuniaux 1966); the use of tritiated chitin (Molano *et al.*, 1977) and the measurement of the release of soluble dye labelled products from dyed chitin derivatives (Wirth and Wolf 1990; Evrall *et al.*, 1990). The latter category involving using soluble oligosaccharide derivatives includes chromogenic and fluorogenic soluble oligosaccharide substrates chitinolytic activity being detected by measuring the intensity of colour or fluorescence respectively.

Individual assays have inherent strengths and weaknesses and the choice of individual assay depends largely on the sensitivity required. The assays that utilise macromolecular chitin either use colloidal chitin, or regenerated chitin prepared by re-acetylation of chitosan (Molano *et al.*, 1977). Both colloidal chitin and regenerated chitin serve as excellent substrates for the detection of bacteria that secrete chitinases when included in agar media. Chitinase activity being visualised as a zone of clearing around colonies. This assay is both simple to set up and inexpensive, but takes several days to produce a result (Gooday 1994). Additionally, this screen only detects production of secreted chitinases and not all chitinolytic bacteria produce such a zone of clearing. For instance Neugebauer *et al.* (1991) describe the chitinolytic activity of *Streptomyces lividans* in liquid culture that is not apparent on solid media. Also the

zones of clearing can either have sharp edges or fuzzy edges leading to difficulties in comparing the diameter of clearing zones between bacterial isolates. Glycol chitin is a soluble modified (partially 0-hydroxyethylated) form of chitin used in some assays, and is produced by reacetylation of glycol chitosan (Araki and Ito, 1975; Trudel and Asselin 1989). This substrate is particularly useful for including in overlay gels since its soluble nature ensures even distribution. Chitinase activity is detected via negative staining with congo red or the fluorescent brightener Calcafluor white MR2 (McBride *et al.*, 1993; Trudel and Asselin 1989). These substances bind to chitin, Calcafluor white being a fluorescent chitin stain that is detected under U.V. light.

The measurement of release of reducing sugars from macromolecular chitin is the method of choice when directly comparing different bacterial chitinases (Franberg and Schnurer, 1994) and can be performed using the reagent p-dimethylaminobenzaldehyde or by the use of the Schale's procedure using potassium ferricyanide and sodium carbonate (Imoto and Yagishita, 1971). These methods rely on the production of the reducing sugar N-acetyglucosamine and activity is measured via the production of a coloured end product. The major drawback with this method is that many chitinases especially bacterial chitinases are exochitinases producing the end product chitobiose. An additional incubation step is therefore required to degrade this disaccharide to reducing sugar, consisting of the addition of N-acetylglucosaminidase in excess.

Assays using tritiated chitin are both rapid and sensitive (Molano *et al.*, 1977), labelled chitin being prepared by reacetylation of chitosan using tritiated acetic anhydride. The assay is based on the fact that the reaction products of chitin degradation are soluble whereas chitin is not. The soluble reaction products are separated from the insoluble chitin and assayed in a scintillation counter.

The use of soluble chito-oligosaccharide derivatives for detection and measurement of chitinolytic activity add an extra dimension to chitinase assays, in that exochitinase, activity can be differentiated from endochitinase activity (McCreath and Gooday 1992; Franberg and Schnurer,1994). These assays depend on the release of either a chromogenic (p-nitrophenol) or fluorescent (4-methylumbelliferyl) group from the chito-oligosaccharide derivative. The particular chromogenic or fluorescent group acts as a single N-acetylglucosamine in these oligosaccharides and whilst being colourless or non-fluorescent when attached to additional N-acetylglucosamines, it is coloured or fluorescent when released. Therefore, for example, the substrate p-nitrophenyl-N-

acetyl-β-D-glucosamine (PNPGlcNAc) consisting of p-nitrophenol linked to a single N-acetylglucosamine is used for detecting chitobiase (N-acetylglucosaminidase) activity, the substrate p-nitrophenyl-β-D-N,N'-diacetylchitobiose ($PNP(GlcNAc)_2$) consisting of p-nitrophenyl linked to a dimer of N-acetylglucosamine is used for detecting exochitinase activity and the minimum sized oligosaccharide that can be used to detect endochitinase activity is p-nitrophenyl-β-D-N,N',N''-triacetylchitotriose ($PNP(GlcNAc)_3$ consisting of a single p-nitophenyl group linked to a trimer of N-acetylglucosamine. The chromogenic substrates are extremely useful in that their cleavage generates a product that is readily assayable in a normal spectrophotometer at 405nm but are very expensive, whereas, the fluorescent chito-oligosaccharides are much more sensitive and therefore cheaper but suffer from the drawback that a fluorescent spectrophotometer is needed to measure the fluorescence. Fluorogenic chito-oligosaccharides derivatives have also been used to detect chitinase activity cytochemically in cells (Manson *et al.*, 1992) as well as chitinase activity of proteins following electrophoresis and isoelectric-focusing (McBride *et al.*, 1993; McNab and Glover, 1991) enabling molecular mass to be directly estimated.

In this section chromogenic and fluorogenic chito-oligosaccharides are used to characterise the chitinase and N-acetylglucosaminidase activities of the S-symbiont of *G. m. morsitans* and to compare this activity with S-symbionts isolated from two other species of tsetse flies; *Glossina palpalis palpalis* and *Glossina austeni*.

4.2 Results

4.2.1 pH profile of N-acetylglucosaminidase, exochitinase and endochitinase activities of the S-symbiont isolated from *G. m. morsitans*.

S-symbionts initially isolated from *G.m. morsitans* (section 2.1) were grown on sheep blood agar under reduced oxygen conditions for 5 days at 26°C as described in section 2.2. The S-symbionts were then harvested and whole cell lysates prepared (section 2.17). The lysates were assayed for N-acetylglucosaminidase, exochitinase and endochitinase activities over a range of pH values with the chromogenic chito-oligosaccharide derivatives PNPGlcNAc, $PNP(GlcNAc)_2$ and $PNP(GlcNAc)_3$ respectively (section 2.23). The assay was incubated overnight (16 hours) at 26°C and optical densities were read at 405nm against controls containing boiled protein. The results (figure 4.1) show that the S-symbiont has activity against all three substrates, greatest activity being against PNPGlcNAc. The activity against PNPGlcNAc was found in a sharp peak between pH 6.5-7, whereas greatest activity against PNP(GlcNAc)2-3 was found over a broad pH range of pH 5-7.5.

4.2.2 Chitinase and chitinase related activity in different strains of S-symbiont

The S-symbiont isolated from *Glossina m. morsitans* displayed activity against PNPGlcNAc, $PNP(GlcNAc)_2$ and $PNP(GlcNAc)_3$ To ascertain if different strains of the S-symbiont had similar activities whole cell extracts from three different species of tsetse were compared; three different strains of S-symbionts were isolated from three different species of tsetse: *Glossina morsitans morsitans, Glossina palpalis palpalis* and *Glossina austeni* as described in section 2.1. They were then further grown on blood agar under reduced oxygen conditions as described in section 2.2. The S-symbionts were harvested from the blood-agar plates after 5 days growth at 26°C and resuspended in nutrient broth to O.D600 of 0.6. Chitinase and N-acetylglucosaminidase activities were then determined for each isolate as described in section 2.17 and 2.23. All of the S-symbiont isolates showed a similar profile of chitinolytic activity. They all had higher N-acetylglucosaminidase activity than exochitinase/endochitinase activity (figure 4.2). The extracts of S-symbiont isolated from *G. m. morsitans* displayed the greatest chitinase and N-Acetyglucosaminidase activities.

4.2.3 SDS-PAGE analysis of chitinase activity of S-symbionts

Extracts of the three strains of S-symbionts prepared as described in section 2.17, were analysed by SDS-PAGE (section 2.18). The proteins were then renatured by washing away the SDS with isopropanol (section 2.19) and the chitinase activity detected by incubating the gel with fluorescent chito-oligosaccharide derivatives (section 2.19) and then visualised on a U.V transilluminator. A number of bands of chitinase activity were detected ranging in size from approximately 35kDa to 125kDa, the major activity was associated with the 125kDa protein. The cell extracts from the three different strains of tsetse had an identical profile of chitinase activity when detected with $4MU(GlcNAc)_3$ (figure 4.3) as when detected with $4MU(GlcNAc)_2$, (data not shown) although they were less visible when detected with $4MU(GlcNAc)_2$ Similar gels treated with 4MU(GlcNAc)4 and 4MUGlcNAc yielded no fluorescent bands when visualised by U.V. radiation (data not shown).

4.2.4 Induction of S-symbiont chitinases.

All the chitinase activity detected in the S-symbionts was of a constitutive nature. To assess whether this chitinase activity could be induced by chitin, the S-symbionts were grown in the presence of a combination of 1% chitin and 1% glycol chitin prepared as described in section 2.22 and 2.26. The culture was incubated for four days at 26°C under reduced oxygen conditions. After growth the chitinase activity was detected as described previously (section 2.23) relative to controls grown in the absence of chitin. No difference in chitinase activity was detected between the S-symbionts grown with or without the presence of chitin (data not shown).

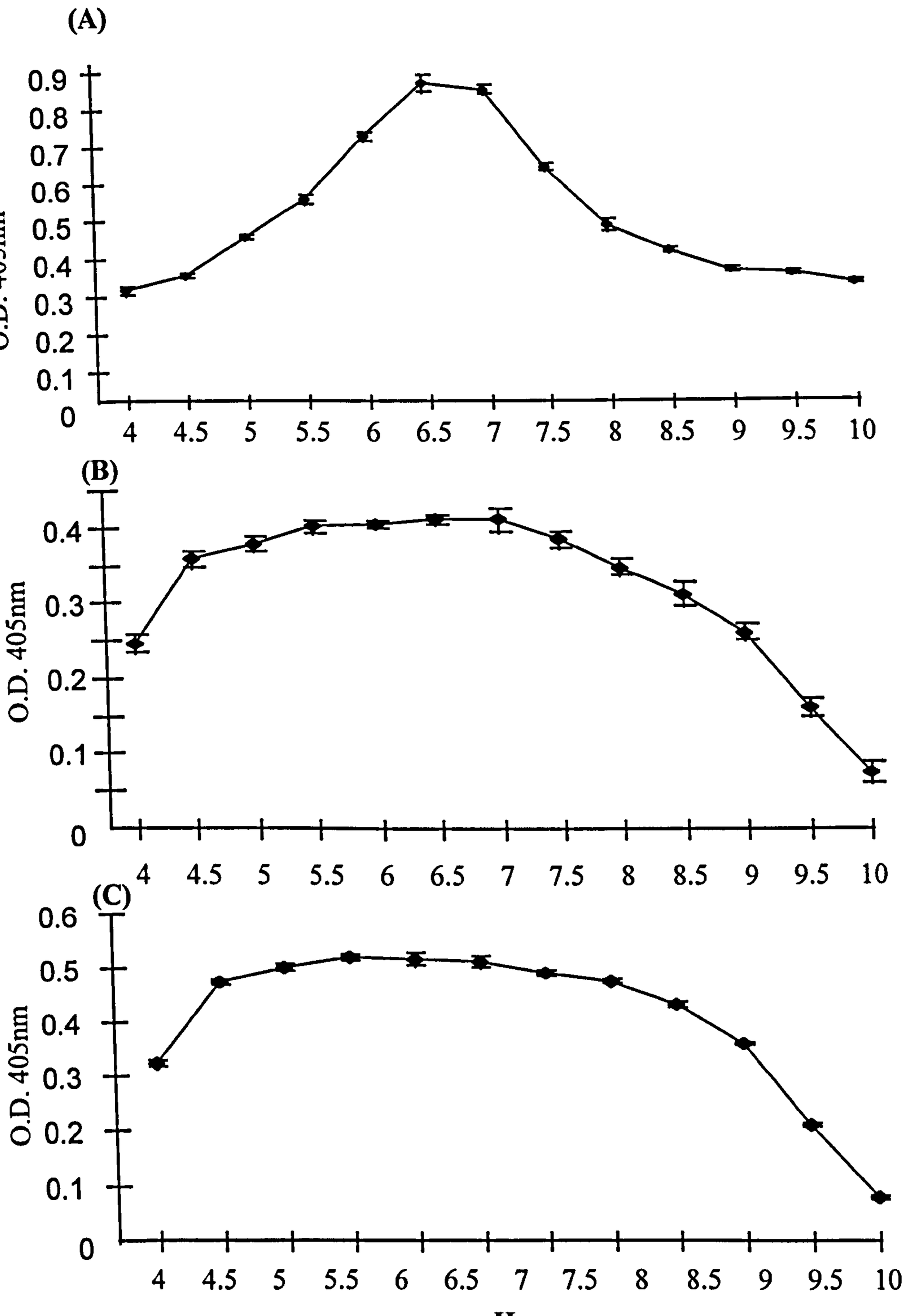


Figure 4.1 pH profile of ; (A) N-acetylglucosaminidase activity, (B) exochitinase activity and (C) endochitinase activities of whole cell extracts of the S-symbiont isolated from *Glossina m. morsitans* as assayed with PNPGlcNAc, $PNP(GlcNAc)_2$ and $PNP(GlcNAc)_3$ respectively.

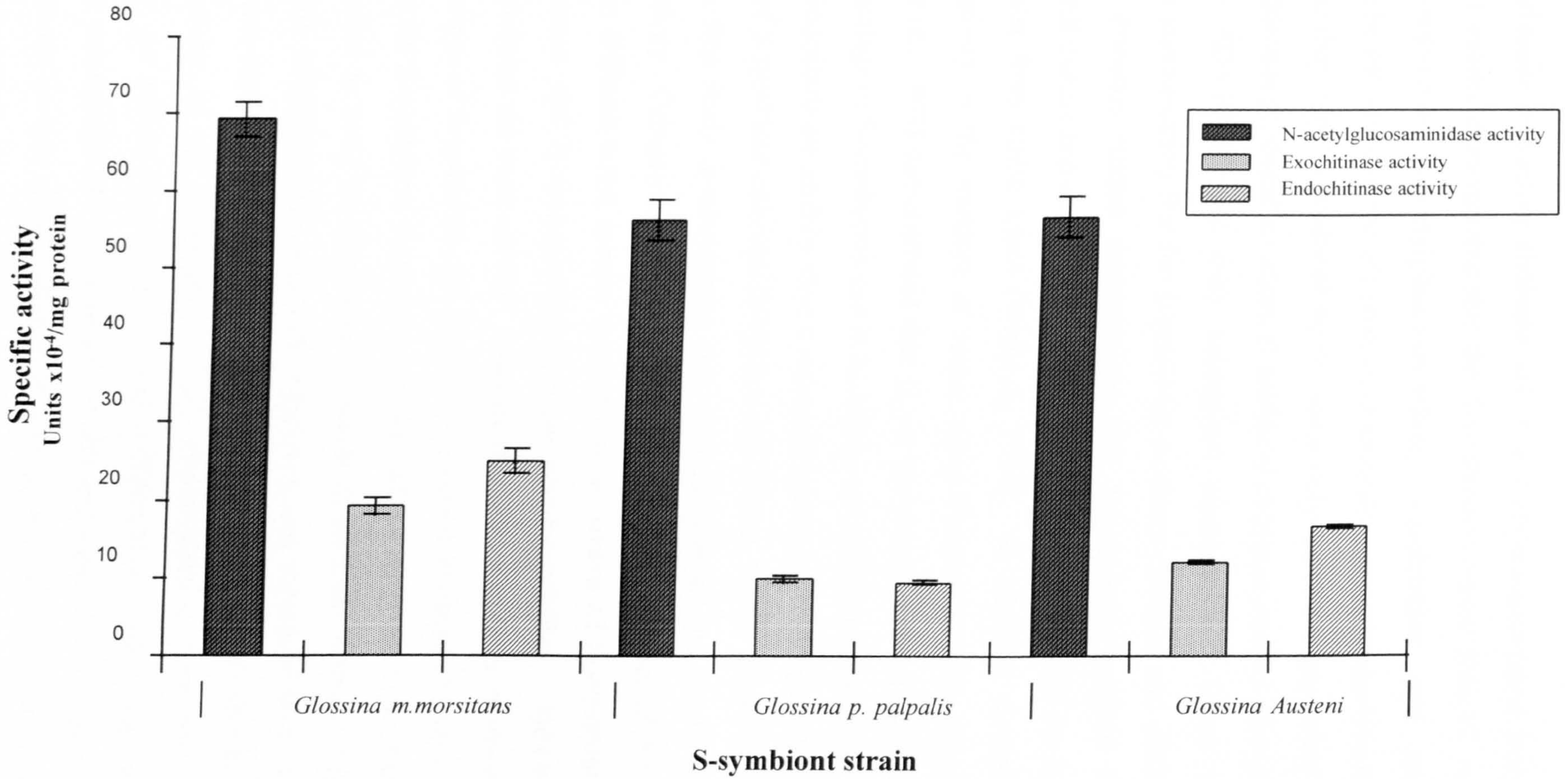


Figure 4.2 Chitinase and N-acetylglucosaminidase activities of whole cell extracts of S-symbionts strains isolated from three species of tsetse fly; *Glossina m. morsitans*, *Glossina p. palpalis* and *Glossina austeni.* Cell extracts were incubated with PNPGlcNAc, $PNP(GlcNAc)_2$ and $PNP(GlcNAc)_3$ to detect N-acetylglucosaminidase, exochitinase and endochitinase activities respectively. Specific activity is expressed as Units $x10^{-4}$ of chitinase/ N-acetylglucosaminidase activity per mg protein, where a unit of activity is defined as the amount of enzyme required to produce one μg of PNP per hour.

PAGE

NUMBERING

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4.3 Discussion

Initial experiments measuring chitinase and N-acetylglucosaminidase activity over a range of pH values, demonstrated that the S-symbiont isolated from *G. m. morsitans* exhibits constitutive N-acetylglucosaminidase, exochitinase and endochitinase activities. The similarity of the pH profiles of exochitinase and endochitinase activities suggests that they may be produced by the same enzyme, this is supported by the fact that both substrates produced an identical profile of chitinase activity after separation of proteins by SDS-PAGE and their subsequent renaturation. These results also demonstrate unequivocally that the S-symbiont produces chitinase and chitinase related activities. Previous studies demonstrated that S-symbionts purified from *Aedes albopictus* cell culture had chitinase activity as measured by release of the dye remazole brilliant violet from chitin azure (Welburn, 1991). In addition, further studies of chitinase activity in the midguts of tsetse using fluorescent chito-oligosaccharides (Welburn *et al.*, 1993) demonstrated that *G. m. morsitans*, a tsetse species known to have high carriage of S-symbionts and to be highly susceptible to trypanosome infection had higher endochitinase activity than a refractory species, *Glossina austeni*, which has a low level of S-symbiont infection (Welburn and Gibson, 1989). However, this present work is the first study demonstrating that pure cultures of the S-symbiont produce chitinase activity. Comparison of chitinase activity between isolates of the S-symbiont derived from different tsetse species showed that all strains of S-symbiont tested had similar chitinase and N-acetylglucosaminidase activity profiles. However, *G. m. morsitans* produced the most activity. It seems therefore that *G. m. morsitans* not only has high carriage of S-symbionts but that these S-symbionts produce more chitinolytic activity than the S-symbionts from other strains. SDS-PAGE analysis of the chitinase activity revealed as many as eight separate bands of chitinase activity (fig 4.3) when incubated with $4MU(GlcNAc)_2$ and $4MU(GlcNAc)_3$ and visualised by U.V. radiation. No activity was detected with the substrate $4MU(GlcNAc)_4$ (data not shown) suggesting that the activity of the chitinase is more exochitinolytic than endochitinolytic. Additionally, no activity was detected with 4MUGlcNAc which suggests that this technique is not suitable for visualisation of this particular N-acetylglucosaminidase possibly because of loss of a co-factor or maybe SDS-PAGE analysis irreversibly damages the protein.

Section 5

Cloning of the chitinase gene <u>chiA</u> of the S-symbiont of <u>Glossina morsitans morsitans</u>.

5.1 Introduction

Section four described the analysis and characterisation of the chitinase activity associated with the S-symbionts of *Glossina morsitans morsitans, Glossina austeni* and *Glossina palpalis palpalis.* All three strains displayed constitutive exochitinase and endochitinase activity, detected using the soluble chito-oligosaccharides 4MU$(GlcNAc)_3$,4MU$(GlcNAc)_2$, PNP$(GlcNAc)_2$ and PNP$(GlcNAc)_3$, as well as constitutive N-acetylglucosaminidase activity assayed with PNP(GlcNAc). In particular, the exochitinase and endochitinase activities were produced by an identical profile of different sized proteins when detected using the respective fluorescent substrates after gel electrophoresis. The proteins displaying chitinase activity ranged in size from approximately 39kDa to 130kDa. This section describes the cloning of the chromosomal gene responsible for this chitinase activity in the S-symbiont.

5.2 Results

5.2.1 Generation of gene banks of S-symbiont DNA

Chromosomal DNA was prepared from a culture of the S-symbiont isolated from *G. m. morsitans* as described in section 2.10.2. The DNA was digested to completion using combinations of two different enzymes :- *Eco*RI/*Hin*dIII, *Pst*I/*Hin*dIII, *Eco*RI/*Pst*I, *Bam*HI/*Sal*I, *Bam*HI/*Hin*dIII, *Eco*RI/*Sal*I. Size fractionated fragments > 2kb, obtained following electrophoretic separation and electro- elution (section 2.12, 2.13), were ligated into the cloning vector pUC18. This cloning vector had previously been digested with corresponding enzymes and treated with calf intestinal alkaline phosphatase to prevent self ligation (section 2.11.3). The recombinant DNA was mini-dialysed and then introduced into electro-competent *E. coli* DH5α (section 2.9.2) by electrotransformation (2.9.4). Bacteria carrying recombinant DNA were selected on nutrient agar containing ampicillin, 100μgml^{-1}, Xgal, 32μgml^{-1}, and IPTG, 0.5mM. Transformation efficiencies of 10^7-10^8 cfu. μg DNA^{-1} were regularly achieved by this method. Recombinants carrying inserted symbiont DNA formed white colonies on this medium and accounted for >99% of the transformants recovered, whereas transformants without inserted DNA formed blue colonies. The transformants were pooled and plasmid DNA was isolated, as described in section 2.10.5. Gene banks were digested with relevant enzymes to show the size range of the genomic inserts (figure 5.1). The number of recombinants in each gene bank was recorded (table 5.1). The gene banks were then screened as described below.

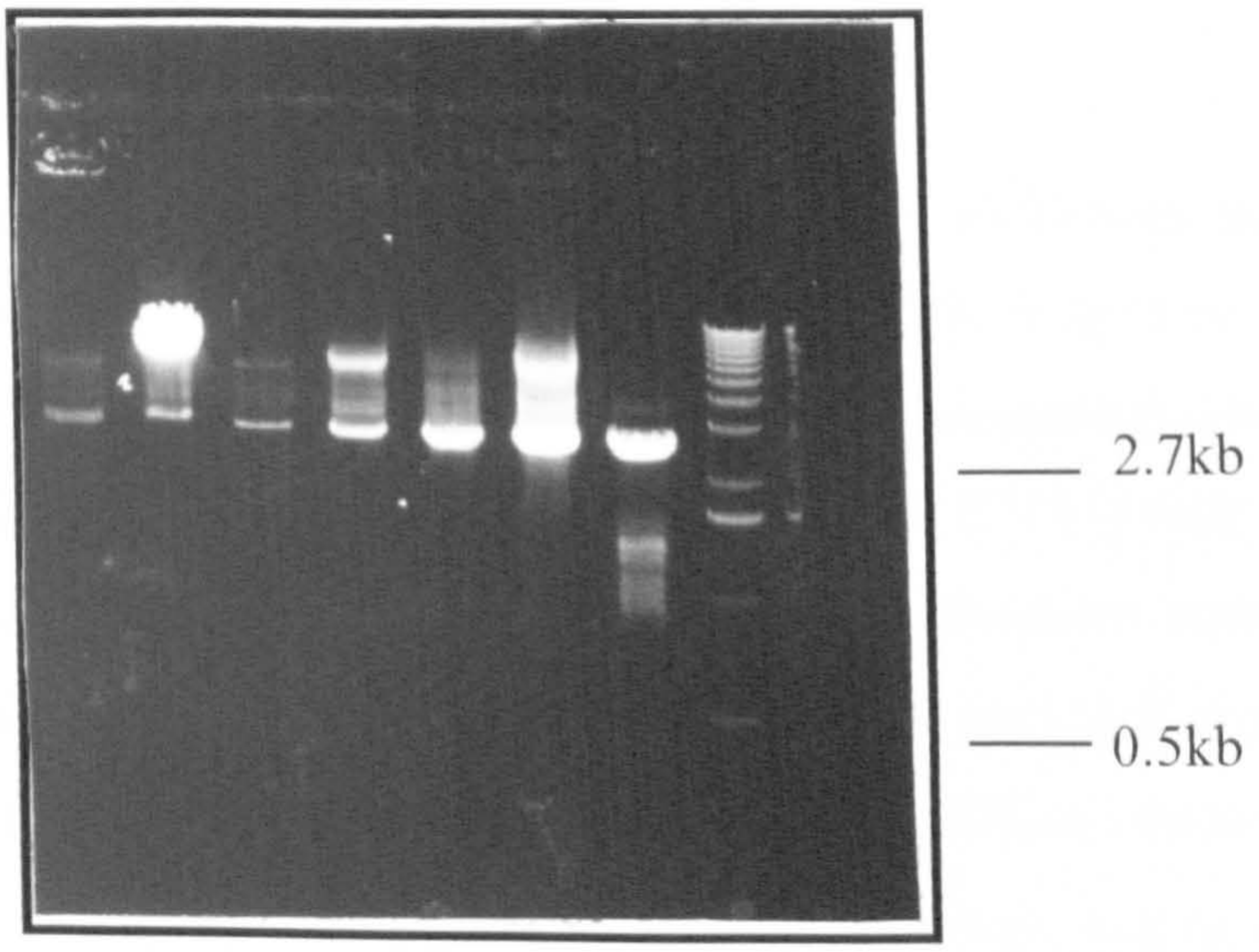


Figure 5.1 Plasmid libraries digested to display size range of genomic S-symbiont DNA. Lanes 1, *Eco*RI/*Hin*dIII library restricted *Eco*RI/*Hin*dIII; 2,*Pst*I/*Hin*dIII library digested with *Pst*I/*Hin*dIII; 3, *Eco*RI/*Pst*I library digested with *Eco*RI/*Pst*I; 4, *Bam*HI/*Sal*I library digested with *Bam*HI/*Sal*I; 5, *Eco*RI/*Sal*I library digested with *Eco*RI/*Sal*I; 6, *Bam*HI/*Hin*dIII library digested with *Bam*HI/*Hin*dIII; 7, *Bam*HI library digested with *Bam*HI. Lane 8 1kb ladder DNA molecular size standard.

Table 5.1 Gene banks of S-symbiont genomic DNA prepared in the cloning vector pUC18.

Restriction endonucleases used to fragment DNA	Size of genomic inserts	Number of individual transformants in gene bank before amplification
*Eco*R1/*Hin*dIII	>2kb	16,500
*Pst*1/*Hin*dIII	>2kb	22,000
*Eco*R1/*Pst*1	>2kb	2,500
*Bam*H1/*Sal*1	>2kb	2,500
*Eco*R1/*Sal*1	>2kb	15,000
*Bam*H1/*Hin*dIII	>2kb	20,000
*Bam*H1	1-1.6kb	20,000

5.2.2 Generation of gene bank from symbiont DNA in LambdaGEM-11 cloning vector

Chromosomal DNA isolated from S-symbiont culture (section 2.10.2) was subjected to limited digestion with *Sau3*A to give a random distribution of DNA fragments in the size range 15-25kb, as described in section 2.11.2. The single strand extensions were partially converted to double-strand form using the Klenow fragment of RNA polymerase with dGTP and dATP as per the supplier's instructions (Promega applications manual p178), and then ligated to pre-prepared λ arms with *Xho*1 overhangs. This approach eliminates the need for size fractionation of genomic fragments, since the partial fill-in reaction prevents insert to insert ligation. The only ligation products possible are single copies of genomic inserts with the λ arms. The products of the ligation reaction were then "packaged" with λ capsid proteins (Promega applications manual p183) and used to infect *E. coli* LE392. The number of plaque forming units per milliliter of the original packaging extract was calculated to be 0.5×10^6 recombinants per μg vector DNA^{-1}.

5.2.3 Cloning of an exochitinase gene

The amplified gene banks prepared in section 5.2.1 were used to transform *E. coli* DH5α which does not produce chitinase. 1μg of pooled, dialysed recombinant DNA was used to transform competent *E. coli* DH5α (section 2.9.4). The transformants were plated on nutrient agar containing ampicillin $100\mu g.ml^{-1}$ and screened as described in section 2.21.1 using the substrates $4MU(GlcNAc)_3$ 4MUGlcNAc. No transformants with 4MUGlcNAc activity were detected, however, ten fluorescent, putative chitinase positive clones were identified from the *Eco*RI/*Hin*dIII gene bank with $4MU(GlcNAc)_3$. The putative positive individual colonies were toothpicked and cultures were incubated at 37°C for 18h into 10ml of nutrient broth containing nalidixic acid, $20\mu g.ml^{-1}$, Each culture was re-screened for $4MU(GlcNAc)_3$ activity as described in section 2.21.2. Of the ten putative positive clones, six remained positive when rescreened (figure 5.2). The six positive clones contained identical plasmids with a 5.2kb insert (figure 5.3). This plasmid was designated pMAT1 (figure 5.4).

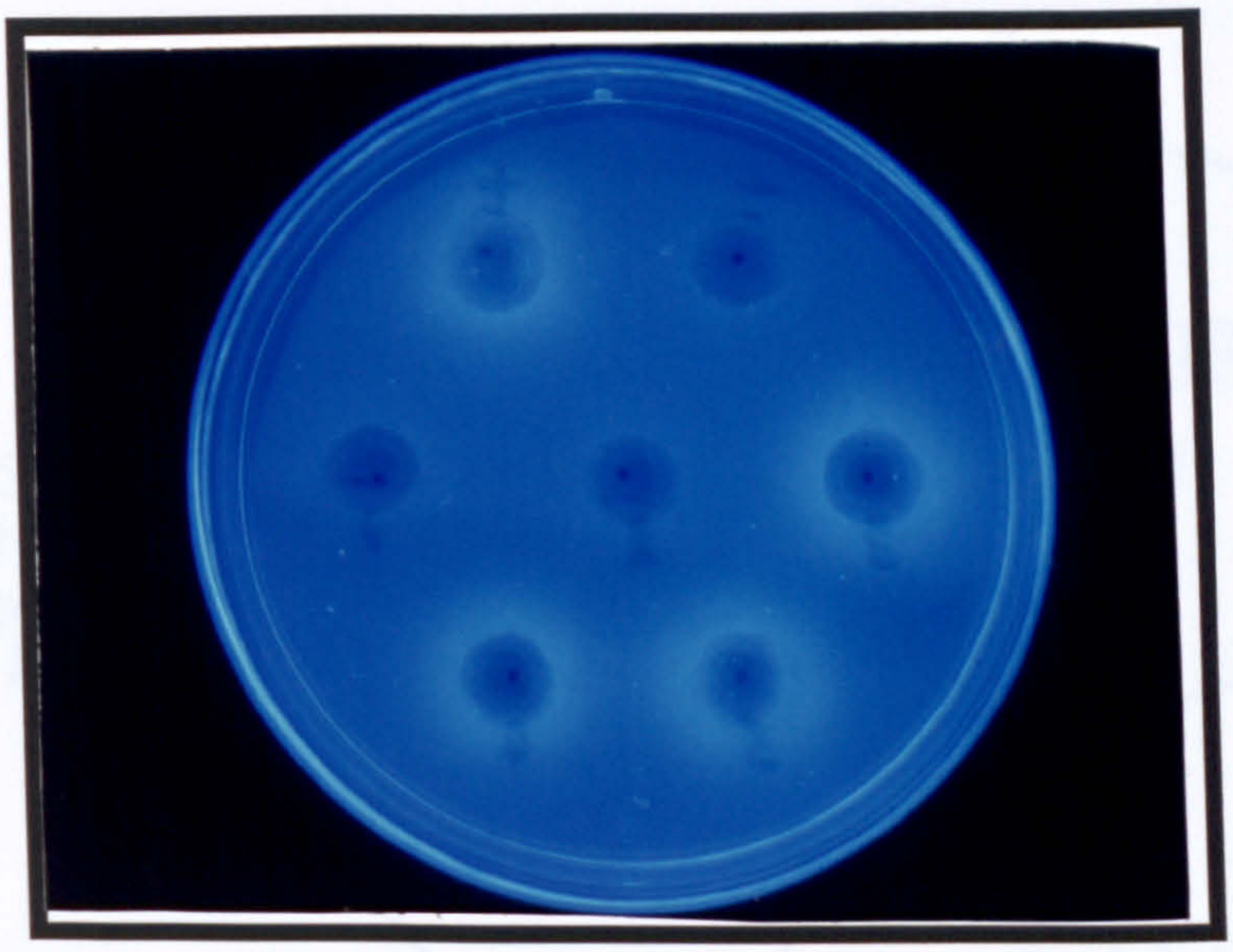


Figure 5.2 Chitinase screen of putative chitinase positive cultures isolated from S-symbiont gene bank.
Cultures were screened directly for chitinase activity by adding 50µl of culture, 20µl of 2% (v/v) toluene in ethanol and 30µl of 50mM TRIS buffer pH 8 to wells punched in minimal agar containing 20µg.ml-1 4MU(GlcNAc)3 and incubation at 37°C for 20 min followed by U.V. transillumination. Positive cultures displayed halos of blue fluorescense surrounding the well..
Well 1: Symbiont culture positive control. Well 2: *E. coli* DH5α culture negative control. Wells 3-5 (left to right): cultures 1-3 respectively (corresponding to lanes 2-4 in figure 5.3). Wells 6&7: cultures 4 and 5 respectively (corresponding to lanes 5 and 6 figure 5.3).

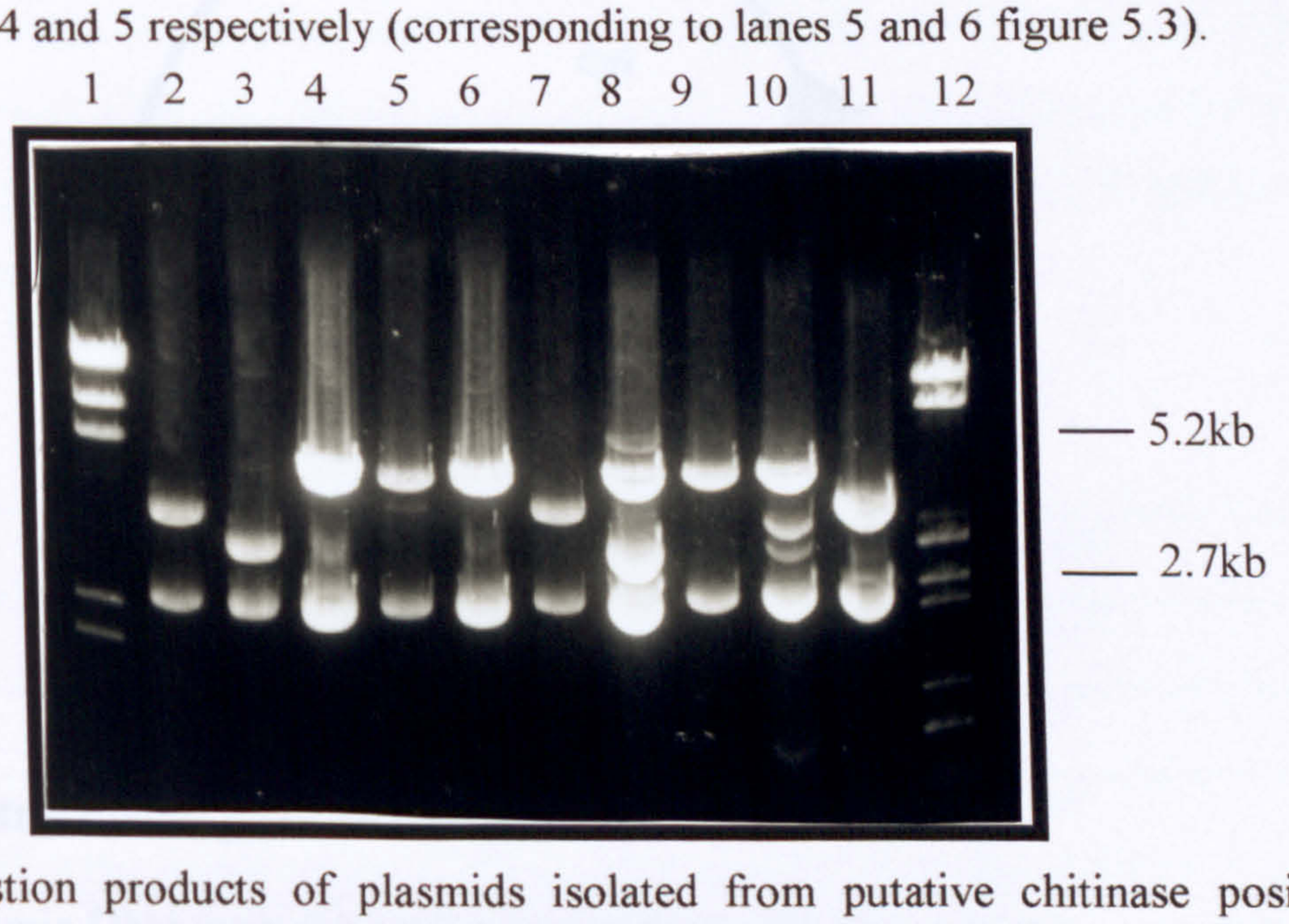


Figure 5.3 Digestion products of plasmids isolated from putative chitinase positive cultures.
Cultures were grown overnight. Plasmids were extracted, digested with enzymes *Eco*RI/*Hin*dIII and analysed by gel electrophoresis. Lanes 1&12 Lambda DNA cut with *Hin*dIII and lambda DNA cut with *Bgl*I, as molecular weight markers respectively. Lanes 2-11 putative chitinase positive cultures 1-10. Cultures containing plasmids depicted in lanes 4-6 and 8-10 displayed chitinase activity when retested, as described in section 2.20.2.

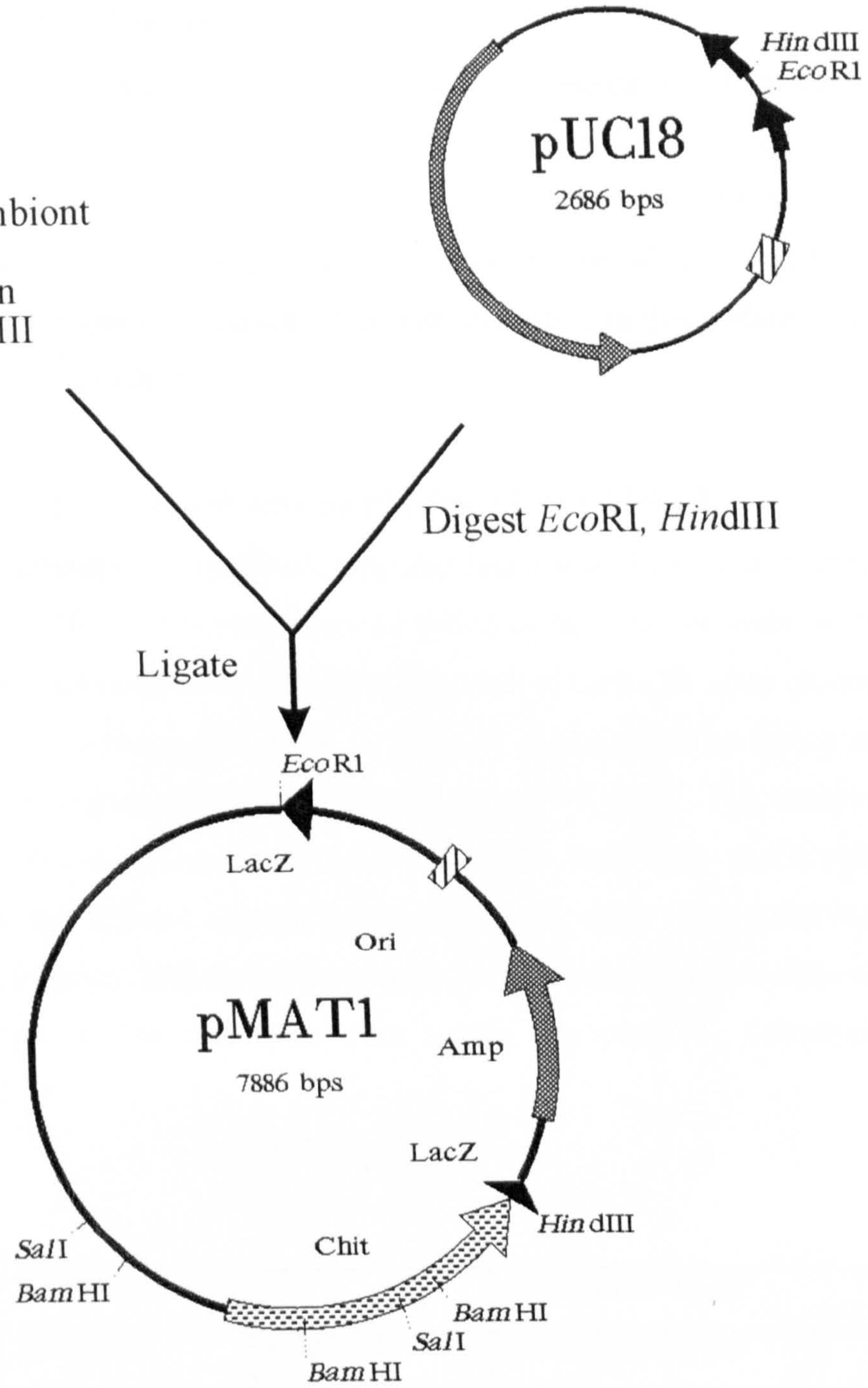


Figure 5.4 Construction of pMAT1

S-symbiont genomic DNA was digested to completion with the restriction enzymes *Eco*RI and *Hin*dIII. DNA fragments >2kb were recovered following gel electrophoresis by electroelution and ligated to compatible ends in pUC18. The ligation products were used to transform *E. coli* strain DH5α and chitinase positive colonies were detected using the fluorogenic chitin substrate 4-methylumbelliferyl β-D-N,N',N''-triacetylchitotrioside, as described in Materials and Methods (section 2.21.1.). The chitinase positive colonies harboured the plasmid pMAT1 containing a 5.2kb insert of S-symbiont DNA.

5.2.4 Back probing of pMAT1 insert

The insert recovered from pMAT1 was used to back-probe chromosomal DNA prepared from the S-symbiont, via southern blotting to confirm its origin (figure 5.5 A and B). The labelled insert hybridised to *Eco*RI/*Hin*dIII restricted S-symbiont DNA of identical size but not to *E. coli* genomic DNA, thus confirming its origin. However, the labelled insert did not hybridise to S-symbiont endogenous plasmids, demonstrating that, in this instance, the chitinase gene is chromosomally encoded.

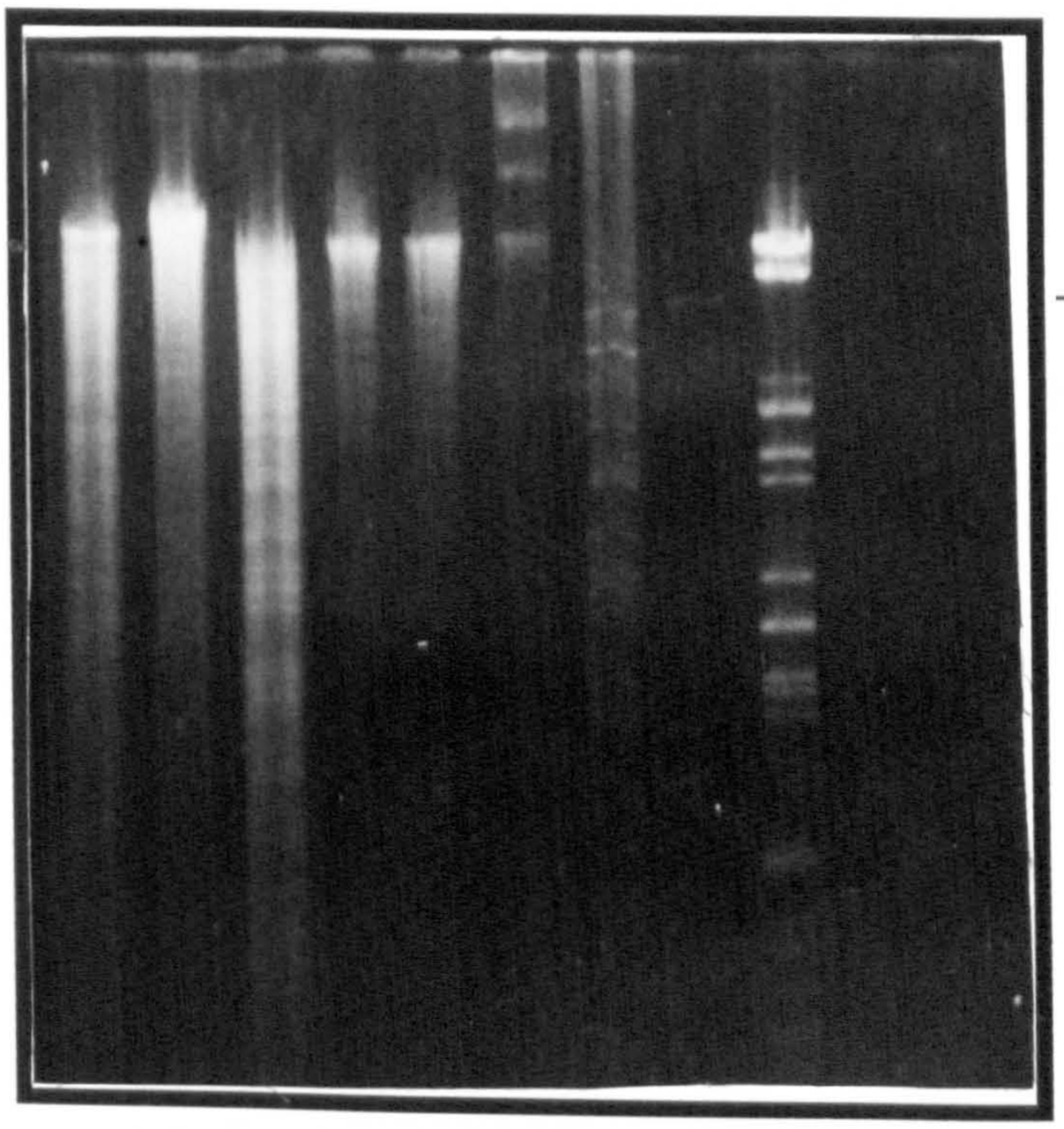
5.2.5 Restriction mapping of the chromosomal insert of pMAT1

The cloning vector pUC18 contains a multiple cloning site (mcs) with 10 unique enzyme sites (figure 5.6) (*Eco*RI and *Hin*dIII enzyme sites are found at the extreme ends of the mcs, therefore cloning DNA fragments into these restriction sites removes all other cloning sites within the mcs from the vector). The insert of pMAT1 was mapped by using the restriction enzymes corresponding to the sites within the mcs of pUC18. The enzymes found to cut the pMAT1 symbiont chromosomal insert were *Sph*I, *Sma*I, *Sal*I, *and Bam*HI (figure 5.7a&b). Enzymes that did not cut this insert were *Sac*I, *Kpn*I, *Xba*I, *and Pst*I. pMAT1 was digested with a variety enzymes that cut once, twice or three times within the insert to map the positions of the restriction sites within the pMAT1 S-symbiont chromosomal insert (table 5.2).

Table 5.2

Single enzyme digests	Double enzyme digests		Triple enzyme digests
*Bam*H1	*Bam*H1/*Sal*1	*Bam*H1/*Sph*1	*Eco*R1/*Hin*dIII/*Bam*H1
*Sal*1	*Bam*H1/*Sma*1	*Bam*H1/*Eco*R1	*Eco*R1/*Hin*dIII/*Sal*1
*Sph*1	*Bam*H1/*Hin*dIII	*Sal*1/*Sph*1	*Eco*R1/*Hin*dIII/*Sph*1
*Sma*1	*Sal*1/*Sma*1	*Sal*1/*Eco*R1	*Eco*R1/*Hin*dIII/*Sma*1
	*Sal*1/*Hin*dIII	*Sph*1/*Sma*1	
	*Sph*1/*Eco*R1	*Sph*1/*Hin*dIII	
	*Sma*1/*Eco*R1	*Sma*1/*Hin*dIII	
	*Eco*R1/*Hin*dIII		

(A)



1 2 3 4 5 6 7 8 9

(B)

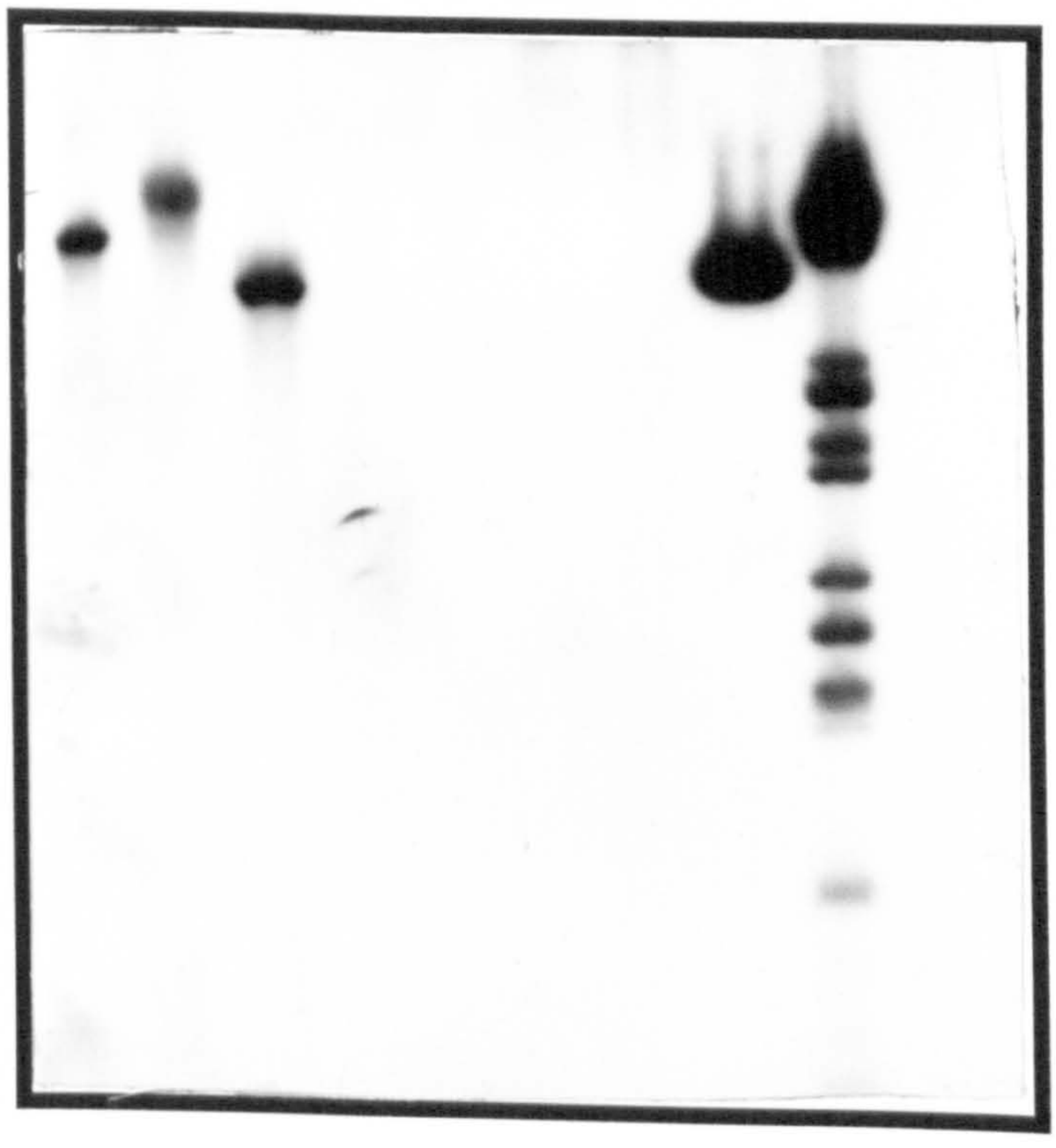


Figure 5.5 Hybridisation of the insert of pMAT1 to S-symbiont (*G.m.morsitans*) chromosomal DNA.

(A) DNA separated in 1% agarose gel; Lanes 1-3, S-symbiont DNA digested with *Pst*I, *Sst*I, and *Eco*RI/*Hin*dIII respectively. Lanes 4-5, *E. coli* chromosomal DNA digested with *Bam*HI and *Hin*dIII, respectively. Lane 6, S-symbiont plasmids uncut. Lane 7, S-symbiont plasmids digested with *Eco*RI/*Hin*dIII. Lane 8, pMAT1 insert. Lane 9, Lambda DNA cut with *Bgl*I, as molecular size standard.

(B) Hybridisation of 5.2kb insert of pMAT1 to southern blot of gel (A).

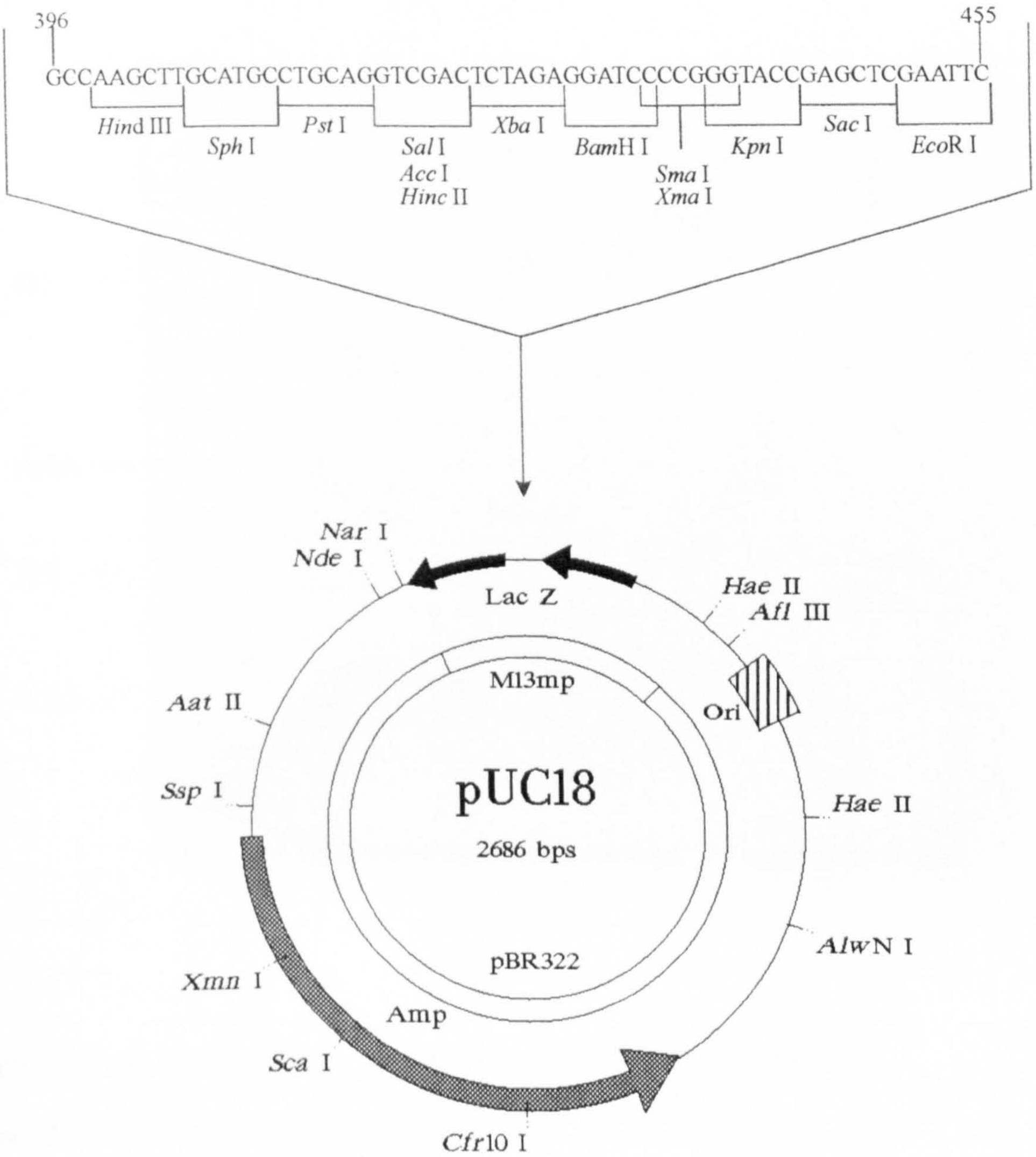


Figure 5.6 Plasmid cloning vector pUC18 with the multiple cloning site enlarged (Pharmacia).

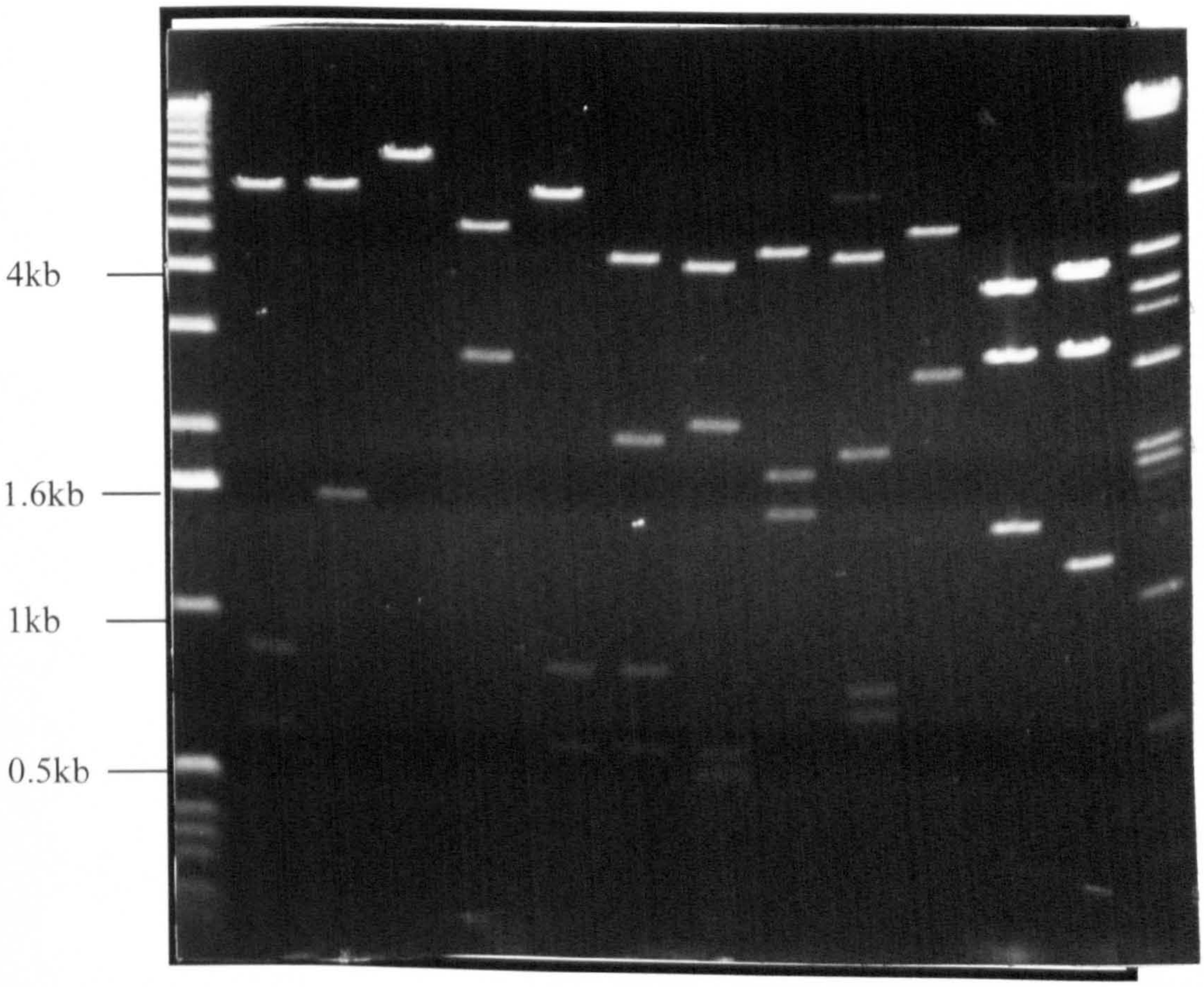


Figure 5.7 (A) Restriction mapping of chromosomal insert of pMAT1
Lane 1, 1kb ladder DNA molecular size marker.
Lanes 2, pMAT1 digested with: *Bam*HI.
Lanes 3, pMAT1 digested with *Sal*I.
Lanes 4, pMAT1 digested with *Sph*I.
Lanes 5, pMAT1 digested with *Sma*I.
Lanes 6, pMAT1 digested with *Bam*HI/*Sal*I.
Lanes 7, pMAT1 digested with *Bam*HI/*Sph*I.
Lanes 8, pMAT1 digested with *Bam*HI/*Sma*I.
Lanes 9, pMAT1 digested with *Sal*I/*Sph*I.
Lanes 10, pMAT1 digested with *Sal*I/*Sma*I.
Lanes 11, pMAT1 digested with *Sph*I/*Sma*I.
Lanes 12, pMAT1 digested with *Eco*RI/*Sal*I.
Lanes 13, pMAT1 digested with *Eco*RI/*Bam*HI.
Lane 14, λ DNA cut with *Cla*I.

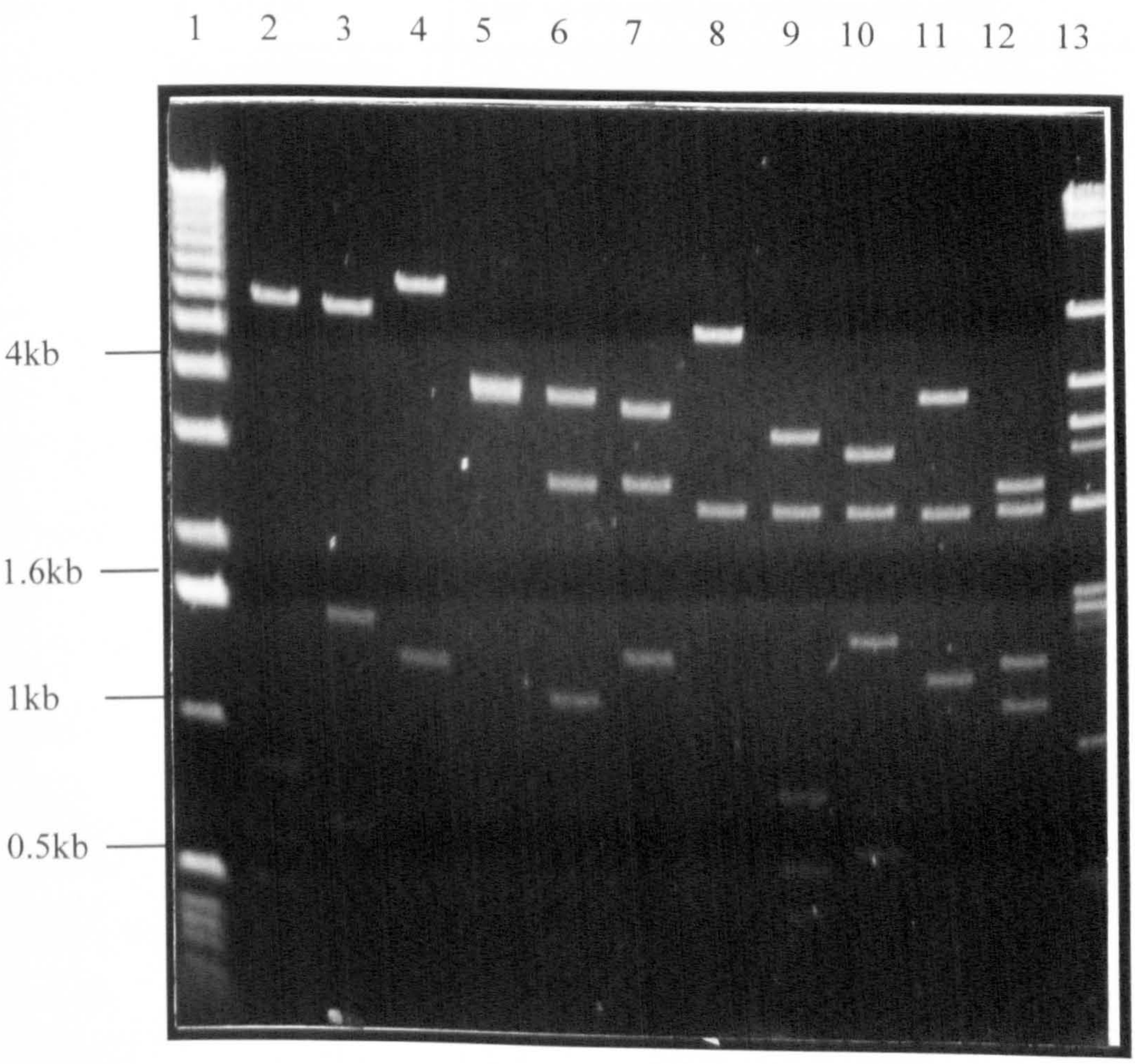


Figure 5.7(B) Restriction mapping of chromosomal insert of pMAT1
Lane 1, 1kb ladder DNA molecular size marker.
Lanes 2, pMAT1 digested with: *Bam*HI/*Hin*dIII
Lanes 3, pMAT1 digested with *Sal*I/*Hin*dIII
Lanes 4, pMAT1 digested with *Sph*I/*Eco*RI
Lanes 5, pMAT1 digested with *Sph*I/*Hin*dIII
Lanes 6, pMAT1 digested with *Sma*I/*Eco*RI
Lanes 7, pMAT1 digested with *Sma*I/*Hin*dIII
Lanes 8, pMAT1 digested with *Eco*RI/*Hin*dIII
Lanes 9, pMAT1 digested with *Eco*RI/*Hin*dIII/*Bam*HI
Lanes 10, pMAT1 digested with *Eco*RI/*Hin*dIII/*Sal*I
Lanes 11, pMAT1 digested with *Eco*RI/*Hin*dIII/*Sph*I
Lanes 12, pMAT1 digested with *Eco*RI/*Hin*dIII/*Sma*I
Lanes 13, λ DNA cut with *Cla*I.

Table 5.3 Restriction fragment sizes of pMAT1 calculated from gels (A) and (B) figure 5.7

Restriction enzyme digest	**Size of restriction fragments (kb)**			
Gel(A) lanes 2-13 respectively				
*Bam*H1	6.5	0.65	0.9	
*Sal*1	6.5	1.6		
*Sph*1	8			
*Sma*1	5.5	2.8		
*Bam*H1/*Sal*1	6	0.9	0.5	
*Bam*H1/*Sph*1	4.6	1.8	0.9	0.7
*Bam*H1/*Sma*1	4.5	1.9	0.65	0.6
*Sal*1/*Sph*1	4.8	1.6	1.4	
*Sal*1/*Sma*1	4.7	1.7	0.8	0.7
*Sph*1/*Sma*1	5.5	2.5		
*Eco*R1/*Sal*1	3.5	2.8	1.4	
*Eco*RI/*Bam*H1	4	2.8	1.2	
Restriction enzyme digest Gel (B) lanes 2-12 respectively				
*Bam*H1/*Hin*dIII	5.7	0.9	0.6	0.5
*Sal*1/*Hin*dIII	5.5	1.6	0.7	
*Sph*1/*Eco*R1	6.5	1.4		
*Sph*1/*Hin*dIII	4.1	3.8		
*Eco*R1/*Sma*1	4.0	2.5	1.2	
*Sma*1/*Hin*dIII	3.8	2.4	1.4	
*Eco*R1/*Hin*dIII	5.2	2.7		
*Eco*R1/*Hin*dIII/*Bam*H1	3	2.7	0.9	0.6, 0.5
*Eco*R1/*Hin*dIII/*Sal*1	2.8	2.7	1.6	0.7
*Eco*R1/*Hin*dIII/*Sph*1	3.8	2.7	1.4	
*Eco*R1/*Hin*dIII/*Sma*1	2.9	2.7	1.4	1.2

5.2.6 Deletion analysis of pMAT1 chitinase clone.

The insert of the recombinant plasmid pMAT1 was subjected to deletion analysis in order to ascertain the position of the gene encoding chitinase activity within the cloned 5.2kb sequence. The restriction map of pMAT1 revealed several restriction sites for the restriction enzymes *Bam*HI, *Sal*I, and *Sma*I. These sites were utilised to delete sections of DNA from the 5.2kb insert carried on pMAT1 (figure 5.8).

5.2.6.1 Deletion analysis utilising BamH1 enzyme sites

Plasmid pMAT1 was digested with *Bam*HI to yield three DNA fragments, 6.5, 0.9 and 0.65kb. The 6.5kb fragment was purified, circularised (figure 5.8) and then used to transform competent *E. coli* DH5α. Transformed bacteria were selected by plating out on agar containing ampicillin,100μg.ml^{-1}, X-gal and IPTG. Several of the resultant white colonies were inoculated to 10ml of nutrient broth containing 100μg.ml^{-1} of ampicillin and incubated with shaking for 18 hrs. Cells were harvested and plasmid DNA was isolated as described in section 2.10.5. That the plasmids recovered carried the desired deletion was confirmed by restriction analysis with the enzymes *Bam*HI, and *Eco*RI/*Hin*dIII. Digestion with these enzyme combinations produced fragments of 6.5kb, and 2.7, 3.8kb respectively, consistent with the expected deletion. One such plasmid was designated pMAT1DB. A cell extract of *E. coli* (pMAT1DB) was then screened for chitinase activity, as described in section 2.21.2, and found not to produce the enzyme.

5.2.6.2 Deletion analysis utilising SalI enzyme sites

Plasmid pMAT1 was digested with *Sal*1 producing two DNA fragments of size 1.6 and 6.5 kb. The largest fragment was purified, circularised and used to transform competent *E. coli* DH5α. Several white colonies obtained after growth on agar containing ampicillin, 100μg.ml^{-1}, IPTG and X-gal were inoculated into 10ml of nutrient broth containing ampicillin at a concentration of 100μg.ml^{-1} and incubated for 18hrs with shaking at 37°C. Digestion of the plasmids isolated from the cells in these cultures by the miniprep method (section 2.10.5) with the enzymes *Sal*I and *Eco*RI/*Hin*dIII produced bands of size 6.5kb and 2.7kb, 3.8kb respectively, consistent with the expected *Sal*I deletion. One such plasmid was designated pMAT1DS. Cell extracts of *E. coli* DH5α (pMAT1DS) were negative for chitinase activity when screened as in section 2.21.2.

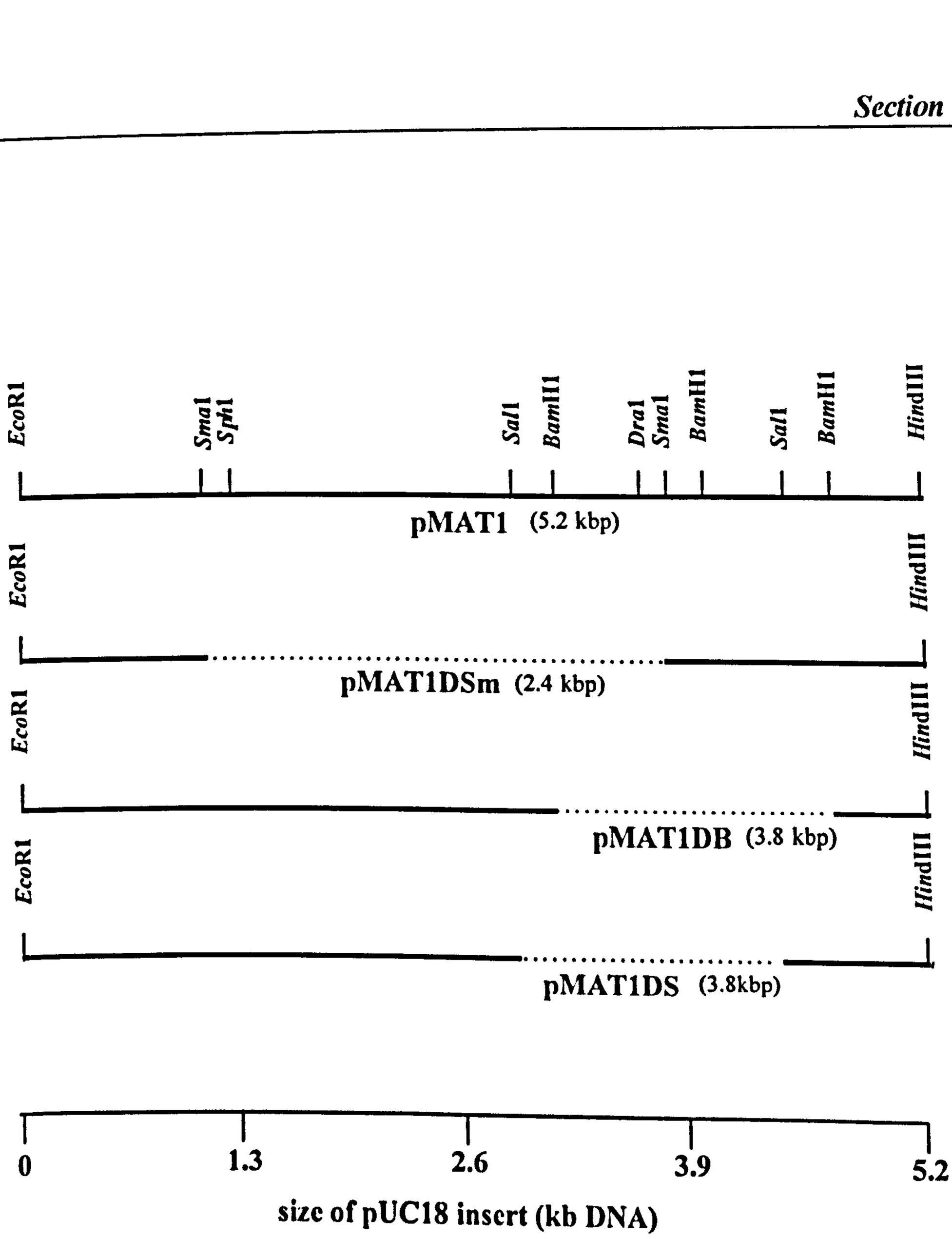


Figure 5.8 Deletion analysis of pMAT1

Deletion analysis was accomplished by deleting fragments of the pMAT1 insert. Deletions were obtained utilising the restriction enzymes *Sma*I, *Bam*HI and *Sal*I, producing the plasmids pMATDSm, pMAT1DB and pMAT1DS, respectively. Bold lines in the figure denote DNA still present within the various inserts and dotted lines represent the sections of pMAT1 DNA that have been deleted.

5.2.6.3 Deletion analysis utilising SmaI enzyme sites

*Sma*I cuts the insert of pMAT1 at two places within the S-symbiont chromosomal insert. Digestion of pMAT1 with *Sma*I produced two fragments of size 2.8 and 5.5kb. The large fragment was purified, circularised and used to transform electro-competent DH5α. The resultant colonies recovered when the transformation mix was plated on nutrient agar containing ampicillin at a concentration of 100μg.ml^{-1}, IPTG and X-gal were cultured and the plasmids isolated. The plasmids were analysed by restriction mapping using *Sma*I and *Eco*RI/*Hin*dIII, producing DNA fragments of 5.5 and 2.7, 2.8kb, respectively, consistent with the *Sma*I deletion. The culture of *E. coli* DH5α containing this plasmid was assayed for chitinase activity (section 2.21.2) and found to be negative. One plasmid was designated pMAT1DS.

5.2.7 Subcloning of pMAT1 for chitinase activity.

Deletion analysis revealed that the DNA encoding chitinase activity within the pMAT1 symbiont chromosomal insert was located, at least in part, towards the *Hin*dIII end of the inserted DNA. To locate the open reading frame (ORF) conferring chitinase activity various subclones were constructed from pMAT1.

5.2.7.1 Construction of SphI/HindIII 3.9kb subclone pMAT1a

Plasmid pMAT1 was cut with the enzymes *Sph*I and *Hin*dIII to produce fragments of size 4.1 and 3.8kb. The fragment of size 3.8kb was electroeluted from the gel (section 2.13) and ligated to pUC18 that had been previously digested with the same enzymes. The ligation products were dialysed and used to transform competent *E. coli* DH5α. Transformants were selected on from nutrient agar containing ampicillin at a concentration of 100μg.ml^{-1}, X-gal and IPTG. Representative colonies were inoculated into 10 mls of nutrient broth containing 100μg.ml^{-1} ampicillin. Plasmids isolated from these cells were analysed by restriction endonuclease digestion. Double digests utilising restriction enzymes *Sph*I and *Hin*dIII produced restriction fragments of 2.7 and 3.8kbp. Triple digests using the restriction enzymes *Hin*dIII/*Sph*I/*Sal*I and *Hin*dIII/*Sph*I/*Bam*HI produced DNA fragments of 2.7, 1.8, 1.6, 0.7 kb and 2.7, 2.1, 0.9, 0.7, 0.5 kb, respectively, consistent with expectations of the desired subclone. The subclone was named pMAT1a (figure 5.9) and was shown to encode a chitinase, when screened as described in section 2.21.2.

5.2.7.2 Construction of SalI/HindIII subclone pMAT1b

The restriction enzyme *Sal*I cuts the insert of the plasmid pMAT1 in two places. Therefore, in order to clone the *SalI/Hin*dIII 2.4kb fragment, pMAT1 was partially digested. This was accomplished by incorporating ethidium bromide into the reaction mix, as described in the methods section 2.10.4. The fragments obtained were separated by agarose gel electrophoresis and the 2.4 kb *SalI/Hin*dIII fragment was purified (section 2.13) and ligated to pUC18 that had been digested with the same restriction enzymes and treated with CIP to prevent self-ligation. The ligation products were used to transform *E. coli* DH5α and the resultant transformants were plated on nutrient agar containing ampicillin at a concentration of 100μg.ml^{-1}. Individual colonies were inoculated to 10ml of nutrient broth containing ampicillin, 100μg.ml^{-1} and were incubated for 18hrs. The plasmids were extracted using the miniprep procedure (section 2.10.5). Digestion of these plasmids with the restriction enzymes *Sal*I/*Hin*dIII and *Sal*I/*Hin*dIII/*Bam*HI yielded DNA fragments of 2.7, 1.6, 0.7kb and 2.7, 0.9, 0.6, 0.5, 0.2, 0.25 kb, respectively, consistent with the restriction map of the expected recombinant. The culture of *E. coli* containing this plasmid, designated pMAT1b, (figure 5.9) produced chitinase, detected as described in section 2.21.2.

5.2.7.3 Construction of SmaI/HindIII 1.7kb subclone pMAT1d

The *Sma*I deletion pMAT1DSm produced in section 5.2.6.3 was restricted with *Sma*I and *Hin*dIII and the digestion products separated by agarose gel electrophoresis. The *Sma*I/*Hin*dIII 1.5kbp fragment was purified and ligated to pUC18 restricted with the same enzymes. White colonies isolated after transformation into *E. coli* DH5α were grown in 10ml broths to saturation. Plasmid DNA was isolated from these cultures and digested with *SmaI/Hin*dIII and *Bam*HI yielding DNA fragments of 2.7, 1.5kb, and 3.5, 0.7 kb, respectively, confirming the subclone. This plasmid was designated pMAT1d. *E. coli* (pMAT1d) was chitinase negative when screened as described in section 2.21.2.

5.2.7.4 Construction of DraI/HindIII subclone pMAT1c

Sequence analysis of the *Eco*RI/*Hin*dIII 5.2 kbp insert of pMAT1 (see section 6) revealed a single *Dra*I restriction site 153bp upstream of the *Sma*I site nearest the *Hin*dIII end of pMAT1. This was utilised to construct the *DraI/Hin*dIII subclone. Plasmid pMAT1 was restricted with enzymes *Dra*I and *Hin*dIII. The reaction products were separated by agarose gel electrophoresis and the fragment of size 1.7kb was purified and ligated to

pUC18 that had previously been digested with *Sma*I and *Hin*dIII. The construct was recovered by transformation of *E. coli* DH5α and its composition was confirmed by restriction digestion with *Dra*I/*Hin*dIII and *Dra*I/*Bam*H1, producing fragments of size 2.8, 0.8, 0.7 kb and 1.5, 1.4, 0.7 (doublet) kb, respectively, as expected. The recombinant plasmid was designated pMAT1c. *E. coli* (pMAT1c) (figure 5.9) and did not produce chitinase, as determined by the screen described 2.21.2.

5.2.7.5 Construction of SalI/BamHI 1.8kbp subclone pMAT1d

Sequence analysis of the *Eco*RI/*Hin*dIII insert of pMAT1 revealed a single *Bgl*I site 1kb upstream from the *Hin*dIII site. This enzyme site was utilised to construct a *Sal*I/*Bam*HI subclone. Plasmid pMAT1 was digested with *Sal*I/*Bgl*I and *Bam*HI/*Bgl*I in two separate reactions and the products separated by agarose gel electrophoresis. Two fragments were recovered, a 500bp fragment resulting from the *Bam*HI/*Bgl*I digest and a 1.3 kb fragment from the *Sal*I/*Bgl*I digest. These fragments were purified and ligated to pUC18 that had previously been digested with *Sal*I/*Bam*HI. The products of the ligation reaction were used to transform competent *E. coli* DH5α and transformants isolated on agar containing ampicillin, 100μg.ml^{-1}. Representative colonies were inoculated into 10ml of nutrient broth containing 100μg.ml^{-1} ampicillin and incubated for 18 h. Plasmids were isolated and characterised by restriction analysis, all transformants carried recombinant plasmids of approximately 4.5kb consistent with the required construction. These plasmids were digested with *Bam*HI, *Bgl*I and *Sal*I to confirm that the *Sal*I/*Bam*HI fragment had been cloned. Digestion with *Bam*HI yielded 3 DNA fragments of size 2.9, 0.9, 0.7 kb and digestion with *Bgl*I and *Sal*I yielded 3 and 2 fragments of size 1.2, 1.5, 1.9 kb and 1.6, 2.9 kb, respectively consistent with expectations. Restriction analysis confirmed the correct fragment of DNA had been cloned since pUC18 is cut twice at positions 245 and 1813 bp outside of the multiple cloning site by *Bgl*I. The culture of this subclone, designated pMAT1e (figure 5.9) did not possess any chitinase activity, as seen when screened as described in section 2.21.2.

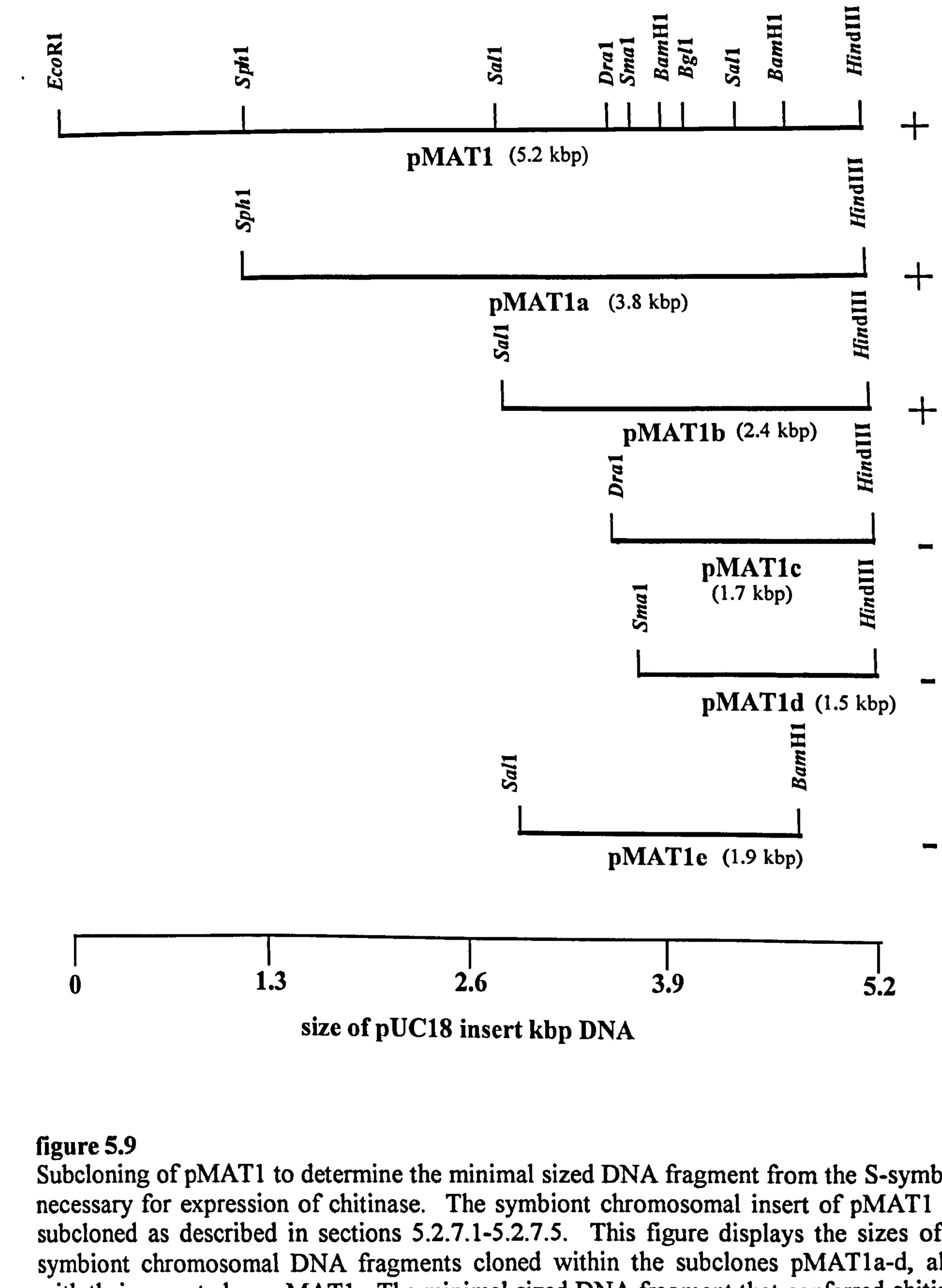


figure 5.9
Subcloning of pMAT1 to determine the minimal sized DNA fragment from the S-symbiont necessary for expression of chitinase. The symbiont chromosomal insert of pMAT1 was subcloned as described in sections 5.2.7.1-5.2.7.5. This figure displays the sizes of the symbiont chromosomal DNA fragments cloned within the subclones pMAT1a-d, along with their parent clone pMAT1. The minimal sized DNA fragment that conferred chitinase activity is the *Sal*I/*Hin*dIII 2.4 kb insert of pMAT1b.

5.2.8 Cloning downstream of the chromosomal insert of pMAT1b

Sequence analysis of the pMAT1b 2.4 kb subclone revealed the presence of an ORF of 1626 nucleotides that starts with an ATG start codon at position 764bp and runs through a *Hin*dIII site used to define one end of the insert (see Section 6). Therefore, it appeared that, despite encoding a chitinase, it was possible that the entire chitinase gene had not been cloned. In order to examine this possibility and to recover the entire chitinase gene if necessary the DNA adjacent to the *Hin*dIII site was cloned and sequenced.

5.2.8.1 Probing of fragmented S-symbiont chromosomal DNA with the insert from subclone pMAT1.8

Chromosomal DNA from the S-symbiont was digested to completion with a number of individual restriction enzymes, including *Sal*I, *Bam*HI and *Eco*RI, in order to identify overlapping pieces of DNA between the pMAT1.8 insert (section 6) and flanking DNA downstream of the chitinase gene. The digestion products were separated by agarose gel electrophoresis, in parallel with molecular size markers and pMAT1.8 (see section 6) restricted with *Bam*HI/*Hin*dIII to serve as a positive hybridisation control. Southern hybridisation analysis was performed on the blotted gel, as described in section 2.15.2, 2.15.4. The gel was probed with labelled insert from pMAT 1.8, as described in section 2.15.1. The probe hybridised to a *Sal*I fragment of 2kb and a *Bam*HI fragment of 1.4 kb (data not shown). These results indicate that further *Sal*I and *Bam*HI enzyme sites are located approximately 1300bp and 900bp downstream of the *Hin*dIII site of pMAT1, respectively.

5.2.8.2 Construction of a BamHI gene bank of symbiont DNA of size 1-1.6kb

The pMAT1.8 insert hybridised with a 1.4kb *Bam*HI fragment of chromosomal DNA adjacent to and overlapping the pMAT 1.8 insert. A gene bank of *Bam*HI digested chromosomal fragments of sizes 1-1.6kb was prepared, as a means of cloning this 1.4 kb fragment carrying the 3' end of the chitinase gene. Genomic DNA (prepared as described in section 2.10.1) was digested to completion with *Bam*HI. DNA fragments between 1 and 1.6 kbp were recovered and purified as described in section 2.13 and ligated to pUC18 that had previously been restricted with *Bam*HI and treated with CIP (section 2.11.3). The ligation mix was used to transform competent *E. coli* DH5α. The transformation mix was plated onto agar containing ampicillin, 100μg.ml^{-1}, X-gal and IPTG. 99% of the resultant

transformants were white colonies. The transformants were pooled and plasmids isolated as described in section 2.10.5.

5.2.8.3 Probing of the BamHI gene bank with the insert of pMAT1.8

The *Bam*HI gene bank, prepared as in section 5.2.8.2, was digested with *Bam*HI and the resulting fragments were separated by gel electrophoresis. The gel (figure 5.10(a)) displayed a range of DNA fragments between 1 and 1.6 kb that had been cloned into pUC18. The gel was blotted, as described in section 2.15.2 and probed with radiolabelled insert of pMAT1.8, as described in section 2.15.1-. The results indicated that the probe had hybridised strongly with a fragment of DNA of size 1.4 kb (figure 5.10(b)), demonstrating that the *BamHI* 1.4kb adjacent/overlapping fragment was present in the *BamHI* gene bank.

5.2.8.4 Isolation of the BamHI 1.4kbp fragment containing the downstream flanking DNA (pMAT2)

Five hundred colonies were screened for the flanking piece of DNA by colony blotting, as described in section 2.15.3. Nylon membranes carrying DNA from lysed colonies were probed with the labelled insert of pMAT1.8 as described in section 2.15.4. Of the 500 colonies screened, 10 individual colonies contained DNA that hybridised with the probe as shown in figure 5.11. These colonies were taken from the master plates and inoculated into nutrient broth containing ampicillin at 100$\mu g.ml^{-1}$. Cultures were incubated at 37°C for 18 hrs. The recombinant plasmids all contained the expected 1.4kb insert (data not shown). One 1.4kb *Bam*HI clone was designated pMAT 122 (figure 5:11)

5.2.9 Restriction mapping of pMAT2

The single clone, pMAT 122 was then used to characterise further the overlapping DNA. The plasmid was digested with the enzymes corresponding to the mcs of pUC18. Only *Hin*dIII, *Sph*I and *Bam*HI had enzyme sites within this DNA fragment (data not shown). Digestion of pMAT122 with *Bam*HI and *Hin*dIII produced fragments of size 1400bp, 2700bp, and 500bp, 3600bp, respectively. Digestion with *Sph*I produced fragments of size 700bp and 3400bp (figure 5.12).

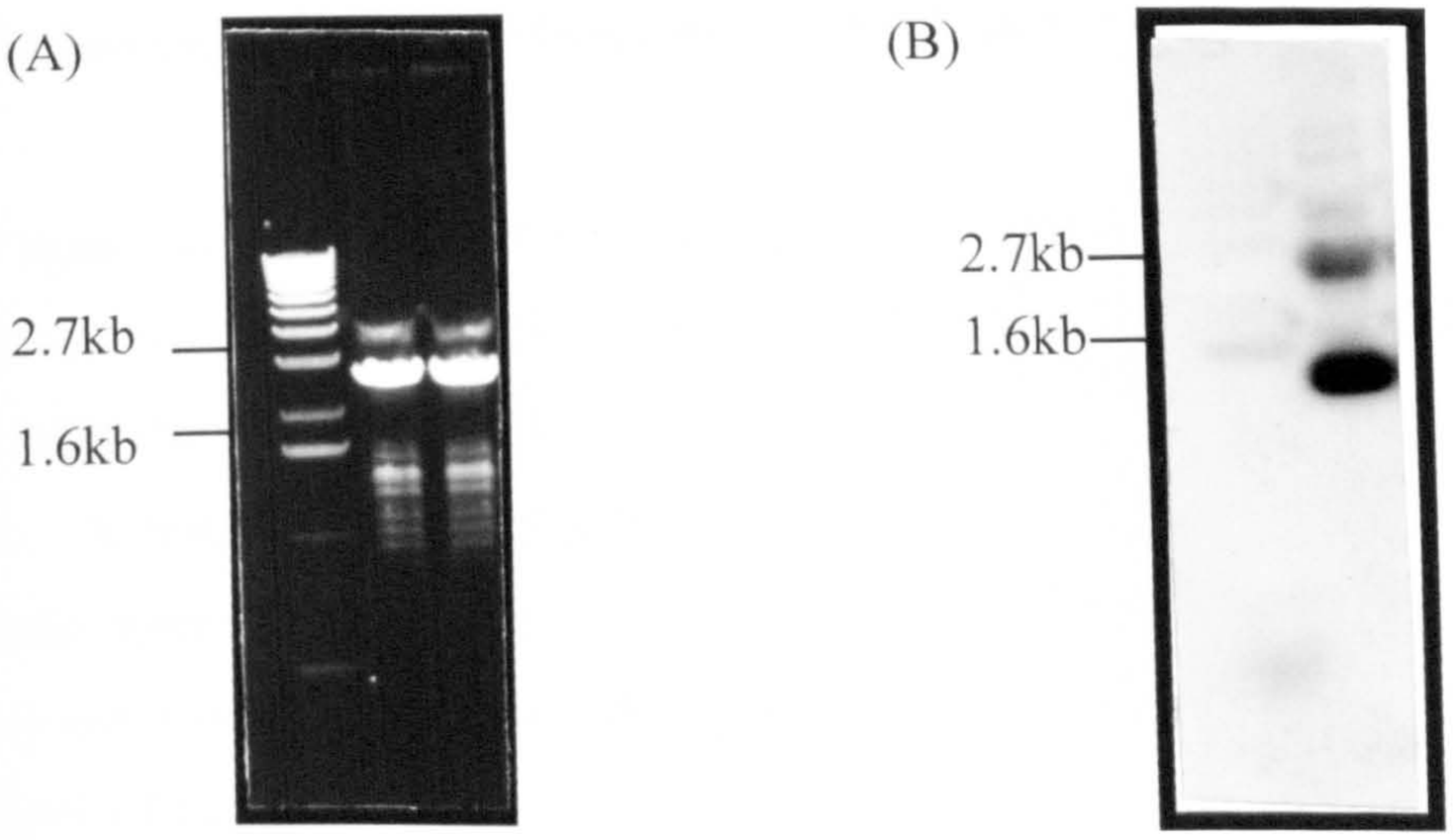


Figure 5.10 Screening of *Bam*HI partial gene bank of S-symbiont DNA
(A) Lane 1, molecular weight marker; Lane 2, *Bam*HI digest of *Bam*HI partial gene bank carrying DNA inserts of sizes 1-1.6kb.
(B) Southern blot analysis of gel (A) probed with the 500bp *Bam*HI/*Hin*dIII insert of pMAT1.8, displaying that the DNA downstream of the chitinase gene is present in this gene bank.

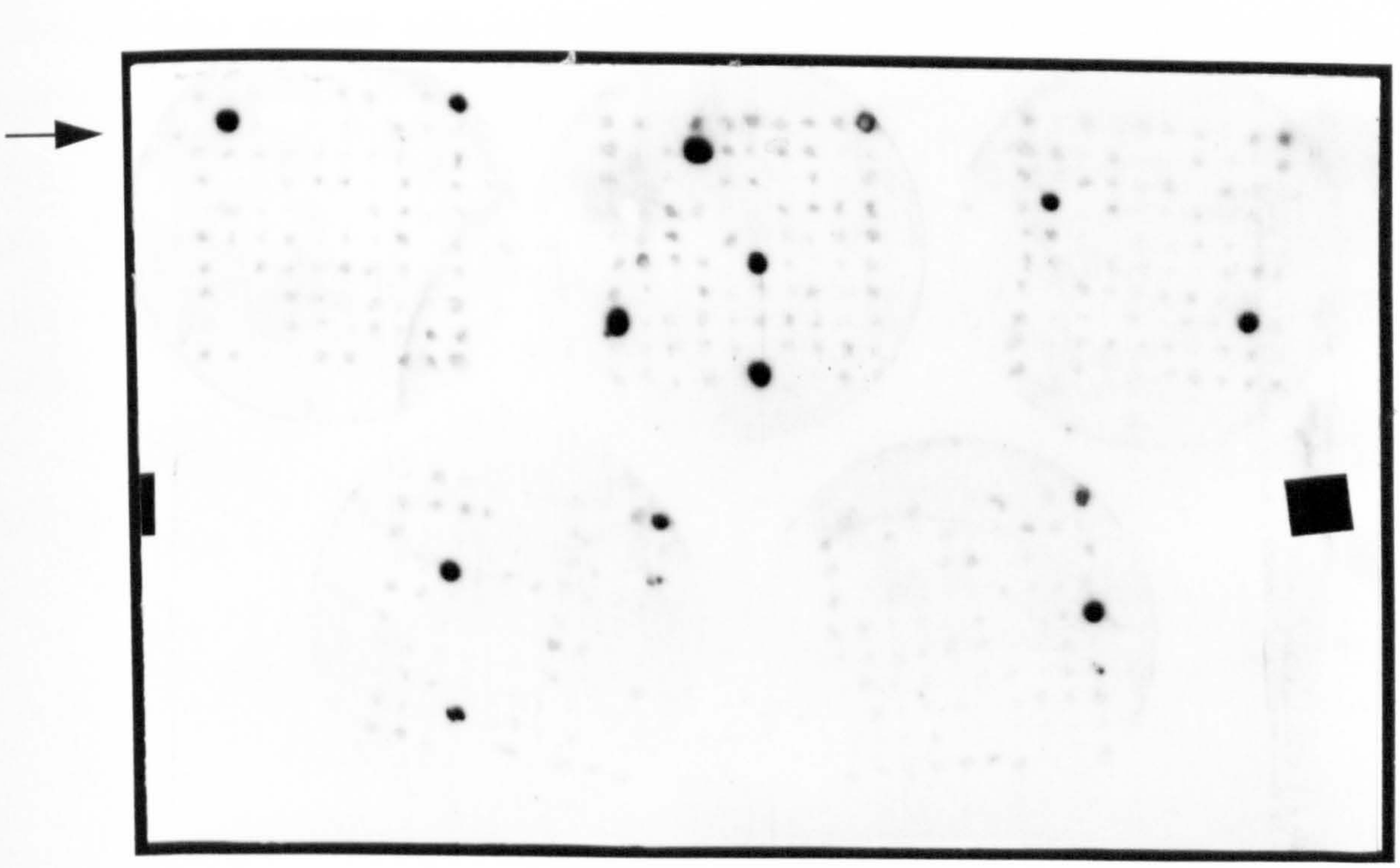


Figure 5.11 Autoradiograph of 500 clones from the *Bam*HI 1-1.6kbp gene bank probed with the insert of pMAT1.8.
Colonies on the top right hand side of each filter are positive control colonies containing pMAT1.8. Negative controls are at top left hand side of each filter. The plasmid isolated from the colony corresponding to the positive signal (arrowed) at position 2,2 filter 1 was named pMAT 122.

5.2.10 Subcloning of adjacent/overlapping fragment pMAT 122

In order to ligate the flanking DNA to the portion of DNA containing the 5' end of the chitinase gene, the insert in pMAT 122 was shortened. pMAT 122 was digested to completion with *Bam*HI and *Hin*dIII and the fragments generated were separated in a 1% agarose gel. A fragment of approximately 900bp was purified and cloned into pUC18 restricted with appropriate enzymes. Restriction analysis of the resulting recombinants with *Bam*HI and *Hin*dIII confirmed that the fragment had been cloned. The subclone was designated pMAT2 (figure 5.12).

5.2.11 Construction of pMAT3, ligation of pMAT1a to pMAT2

Plasmids pMAT1a and pMAT2 were digested with *Sph*I/*Hin*dIII and *Bam*HI/*Hin*dIII, respectively. DNA fragments of 900bp from pMAT2 and 3.8kbp from pMAT1a were excised from a 1% agarose gel, purified and ligated together with pUC18 that had previously been digested with *Sph*I/*Bam*HI. The ligation mix was used to transform competent *E. coli* DH5α. Transformants were isolated on agar containing ampicillin, 100μg.ml-1, X-gal and IPTG. Plasmids extracted from the transformants (section 2.10.5) were digested with *Eco*RI/*Hin*dIII to reveal the size of inserted DNA. Of the 10 transformants screened, one had an insert of the correct size and the desired restriction profile. The plasmid constructed from pMAT1a and pMAT2, was named pMAT3 (figure 5.12). Plasmid pMAT3 DNA was digested in parallel with digests of pMAT 122 and pMAT1a to confirm the correct ligation (figure 5.13).

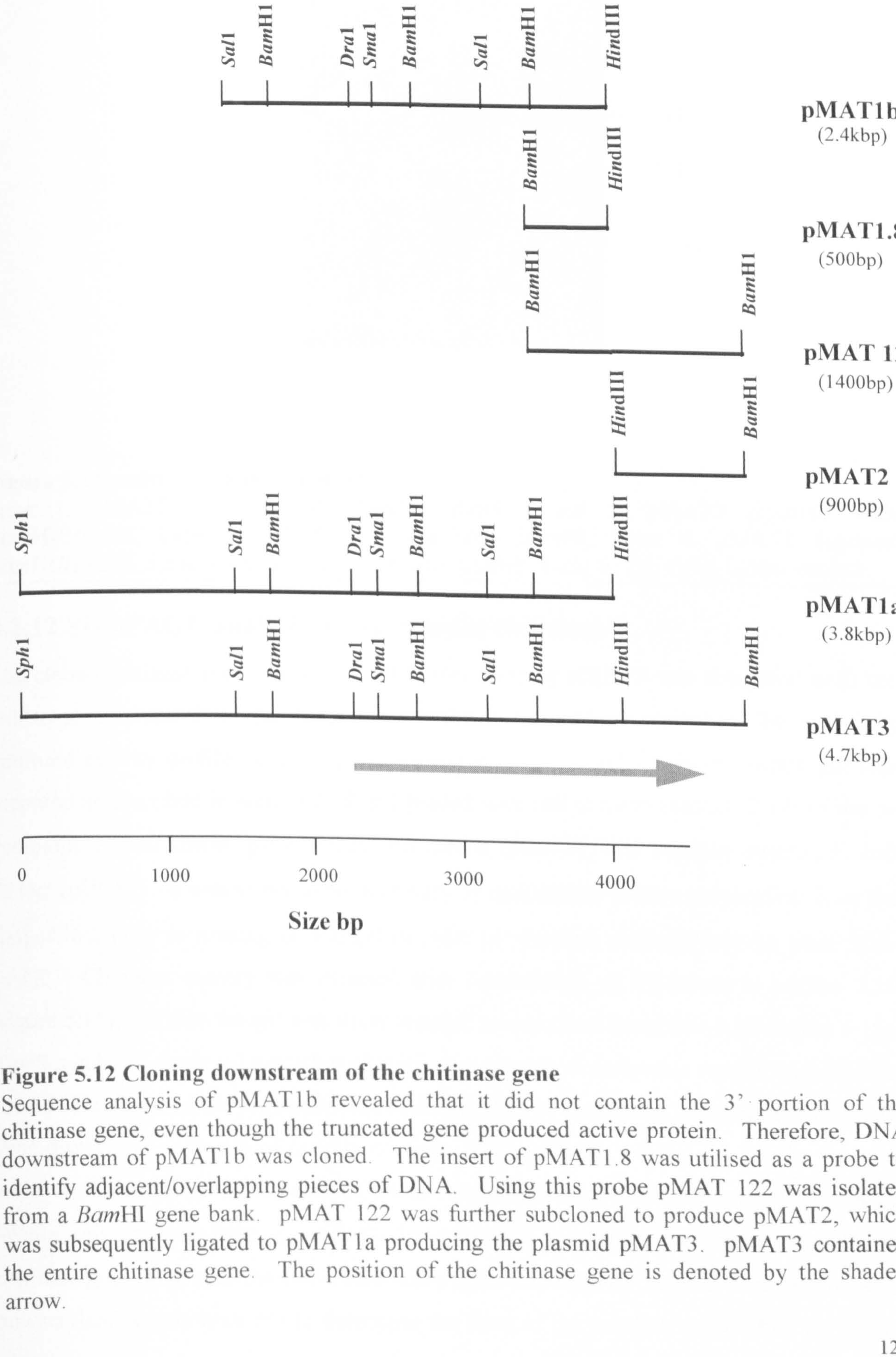


Figure 5.12 Cloning downstream of the chitinase gene

Sequence analysis of pMAT1b revealed that it did not contain the 3' portion of the chitinase gene, even though the truncated gene produced active protein. Therefore, DNA downstream of pMAT1b was cloned. The insert of pMAT1.8 was utilised as a probe to identify adjacent/overlapping pieces of DNA. Using this probe pMAT 122 was isolated from a *Bam*HI gene bank. pMAT 122 was further subcloned to produce pMAT2, which was subsequently ligated to pMAT1a producing the plasmid pMAT3. pMAT3 contained the entire chitinase gene. The position of the chitinase gene is denoted by the shaded arrow.

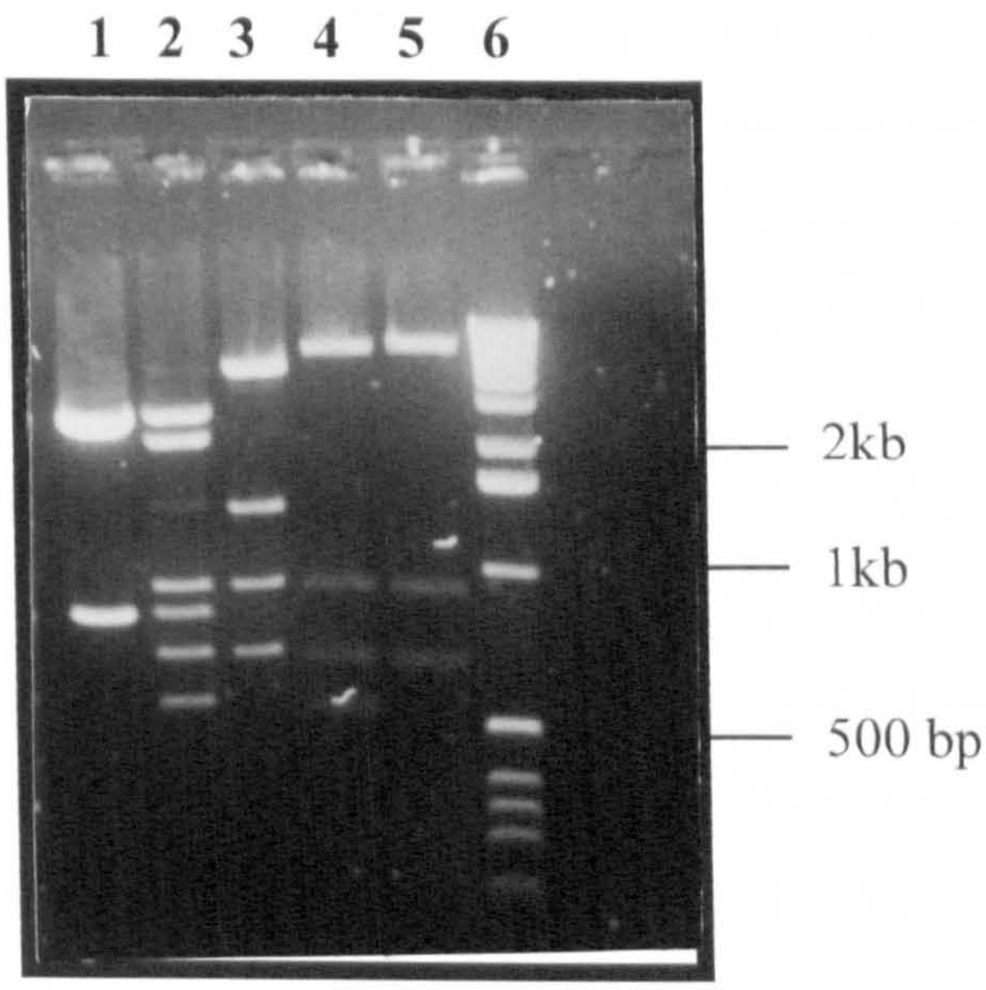


Figure 5.13 restriction analysis of pMAT3
Lane 1, pMAT2 digested with *Bam*HI/*Hin*dIII; Lane 2, pMAT3 digested with *Bam*HI/*Hin*dIII; Lane 3, pMAT3 digested with *Bam*HI; Lane 4, pMAT1 digested *Bam*HI/*Hin*dIII; Lane 5, pMAT1 digested with *Bam*HI. Lane 6, 1kb DNA ladder marker.

5.2.12 SDS-PAGE analysis of S-symbiont chitinase

The cloned chitinase produced in *E. coli* DH5α carrying pMAT3 was compared with the wild-type chitinase from the S-symbiont isolated from *G. m. morsitans* by analysing chitinase activity profiles in SDS polyacrylamide gels. A 10% polyacrylamide gel was prepared as described in section 2.18 and loaded with cell extracts (section 2.17) of the S-symbiont, *E .coli* DH5α (pMAT3), *E. coli* DH5α (pMAT1) and negative control, *E. coli* DH5α (pUC18). It was found to be necessary to concentrate protein preparation from the S-symbiont prior to running on the gel in order to visualise chitinase activity after SDS-PAGE. Chitinase activity was detected after renaturation, as described in section 2.19 (figure 5.14), and then the gel was silver stained, as described in section 2.20 (figure 5.15). The S-symbiont displayed a number of chitinases ranging in molecular weight from 39 kDa to 130 kDa. The sample prepared from *E. coli* (pMAT1) containing the truncated chitinase gene displayed four bands of activity of sizes 39kDa, 43kDa, 47kDa and 50kDa, three of which are identical in size to bands of minor chitinase activity in the wild-type S-symbiont. Whole cell extracts from *E. coli* DH5α (pMAT3) containing the entire S-symbiont chitinase gene, displayed the entire chitinase profile displayed by the source organism. 8% polyacrylamide gels were run to determine the sizes of the proteins constituting the bands

with chitinase activity. This was achieved by marking the positions of the various bands of chitinase activity (after SDS-PAGE and renaturation) by puncturing the gel adjacent to each band of chitinase activity. The gels were then silver stained and the sizes of the bands deduced by reference to the molecular size markers run on the same gel.

The difference in size between the predicted molecular weight of the cloned chitinase (76kDa) and the largest band of chitinase activity detected after SDS-PAGE, 130kDa, suggests that the large band may be a heterodimer. Further gels were run of periplasmic preparations of *E. coli* DH5α (pMAT3) treated in three different ways; boiled with mercaptoethanol, boiled without mercaptoethanol and samples that were not boiled. The results indicate that boiling for 5 minutes caused a shift in chitinase size from 130kDa to 75kDa and that the addition of mercaptoethanol had no further effect on the size of chitinase (figure 5.16).

5.2.13 Profile of chitinase activity of pMAT3

A whole cell extract of *E. coli* DH5α (pMAT3) was prepared as described in section 2.17. This extract was then assayed for chitinase and N-acetylglucosaminidase activities as described earlier (section 2.23). The results showed that the chitinase gene within pMAT3 encodes a chitinase with exochitinase and endochitinase activities and that the exochitinase activity is greater than its endochitinase activity (figure 5.18)

5.2.14 The effect of pH on S-symbiont chitinase activity.

The activity of the cloned chitinase at different pH values was assayed as described in the Materials and Methods, section 2.27. The results indicate that maximum activity against $PNP(GlcNAc)_2$ is found over a broad pH range from pH5.5-7.5 (figure 5.19(B)). The activities at pH 3.5 and pH 10 were approximately 15% of the maximum activity at pH7.

5.2.15 Heat stability of S-symbiont chitinase.

The heat stability of the chitinase was determined as described in section 2.25

The results are displayed in figure 5.19(A) and show that the chitinase is stable up to 48^0C, but thereafter is rapidly denatured.

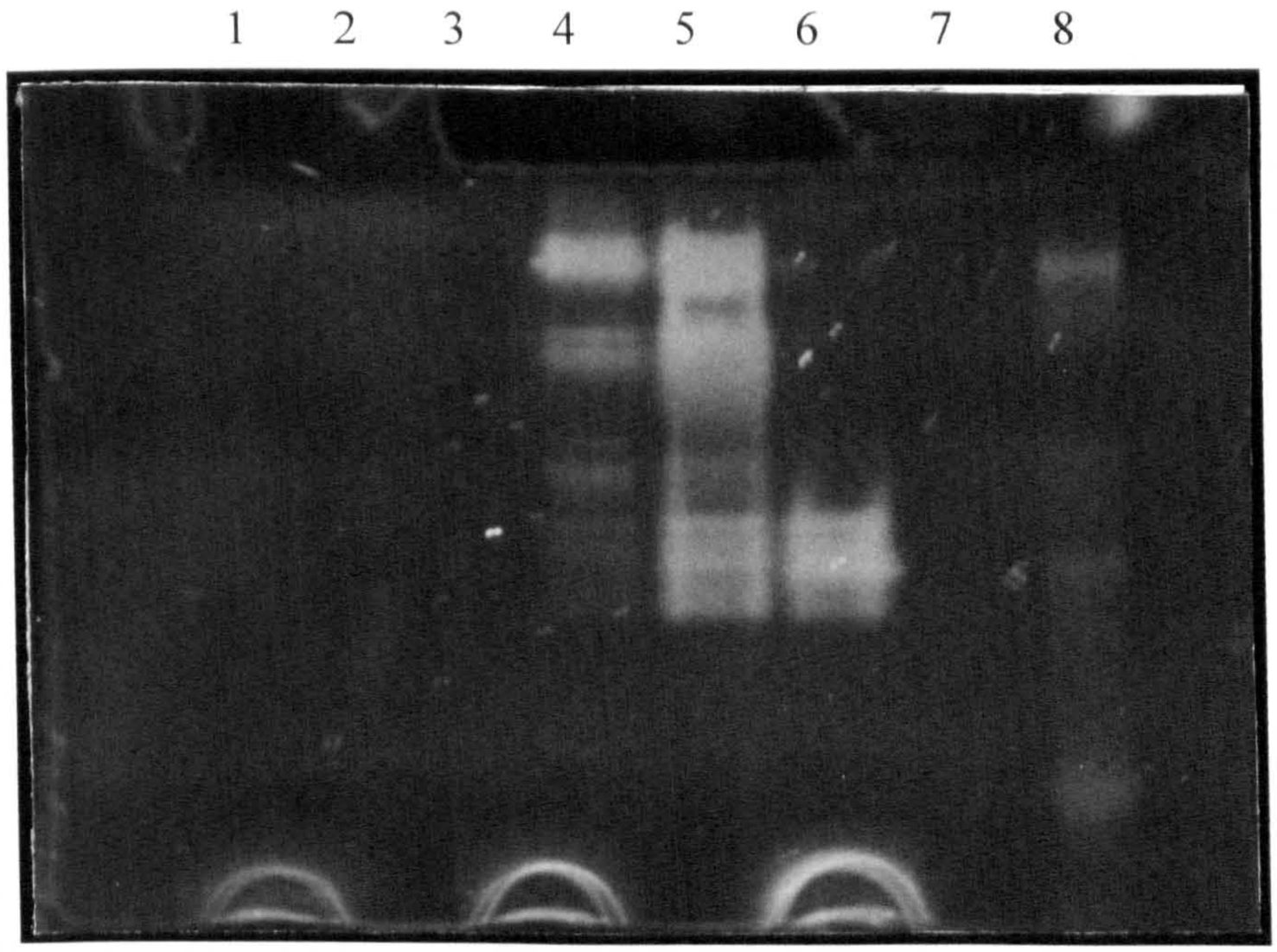


Figure 5.14 Chitinase activity after SDS-PAGE

Lanes 3-6 contain periplasmic preparations of :- Lane 3, *E. coli* DH5α (pUC18); Lane 4, S-symbiont isolated from *G. m. morsitans*; Lane 5, *E. coli* DH5α (pMAT3) containing the entire S-symbiont chitinase gene; Lane 6, *E. coli* DH5α (pMAT1) containing the truncated chitinase gene. Lane 8, contains Biorad prestained molecular size standards. The gel was activity stained with 4-methylumbelliferyl$(GlcNAc)_3$.

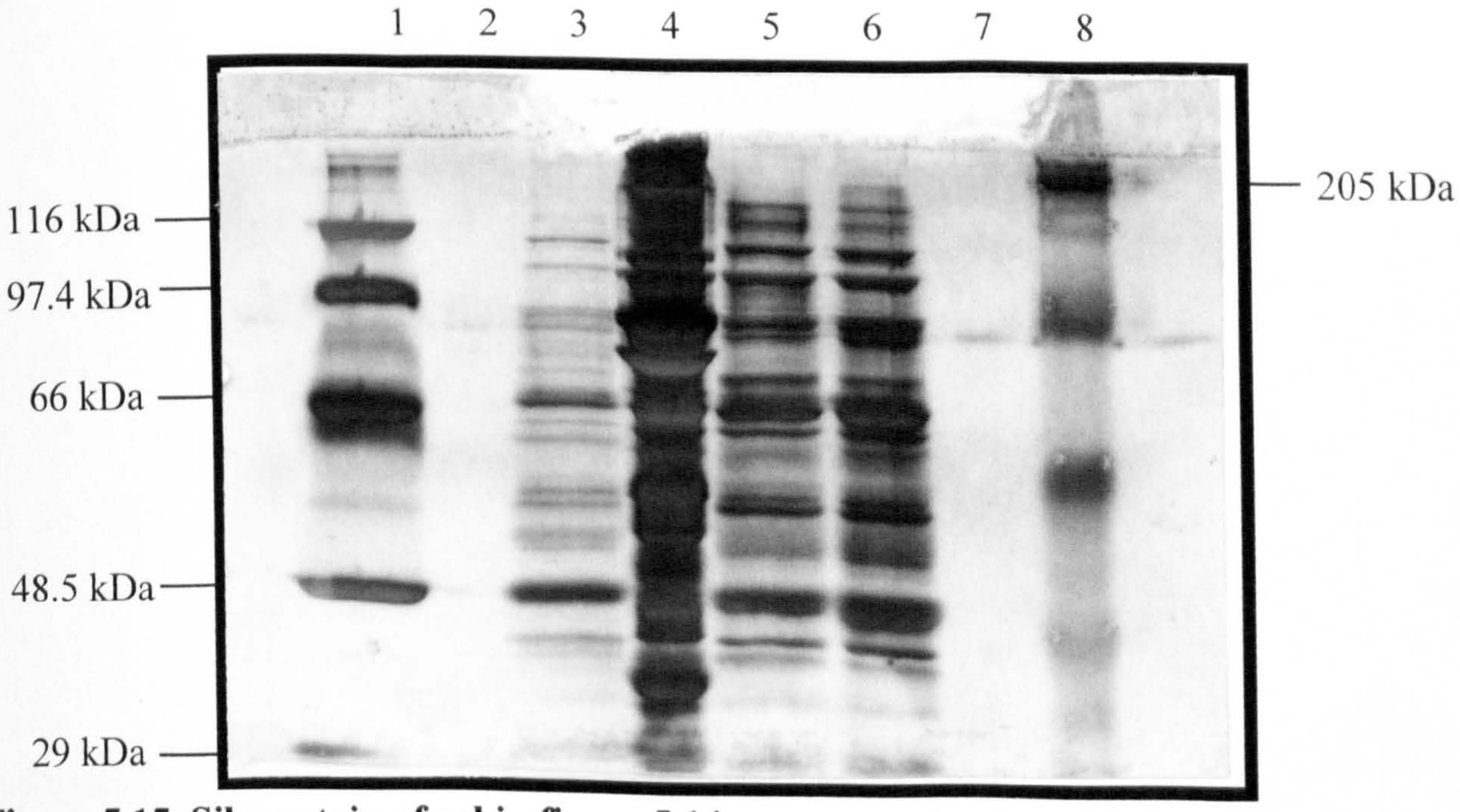


Figure 5.15 Silver stain of gel in figure 5.14

Lane 1, molecular weight marker; Lanes 3-6, equivalent of lanes 3-6 in figure 5.14; Lane 8, Biorad prestained molecular weight marker (Lanes 2 and 7 are blank).

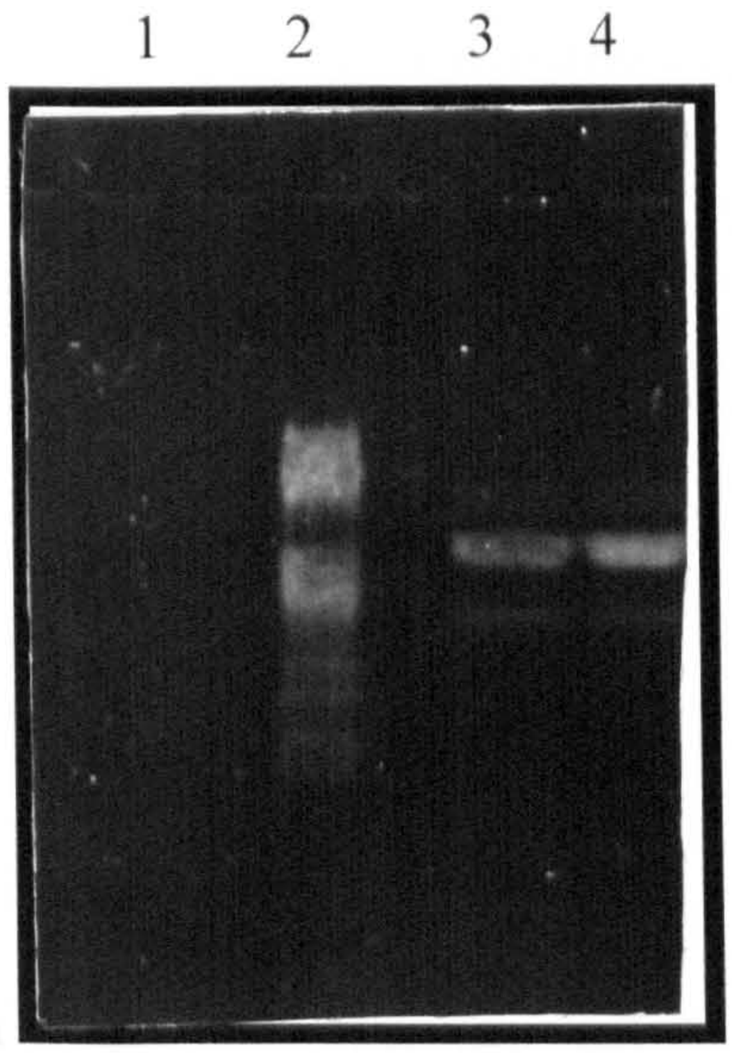


Figure 5.16 Effect of boiling recombinant chitinase, in the presence or absence of mercaptethanol.

Lane 1, Molecular weight markers; Lane 2, DH5α (pMAT3) unboiled; Lane 3, DH5α (pMAT3) boiled for 5 minutes; Lane 4 DH5α (pMAT3) boiled for 5 minutes with mercaptoethanol. The gel was activity stained with 4-methylumbelliferyl$(GlcNAc)_3$.

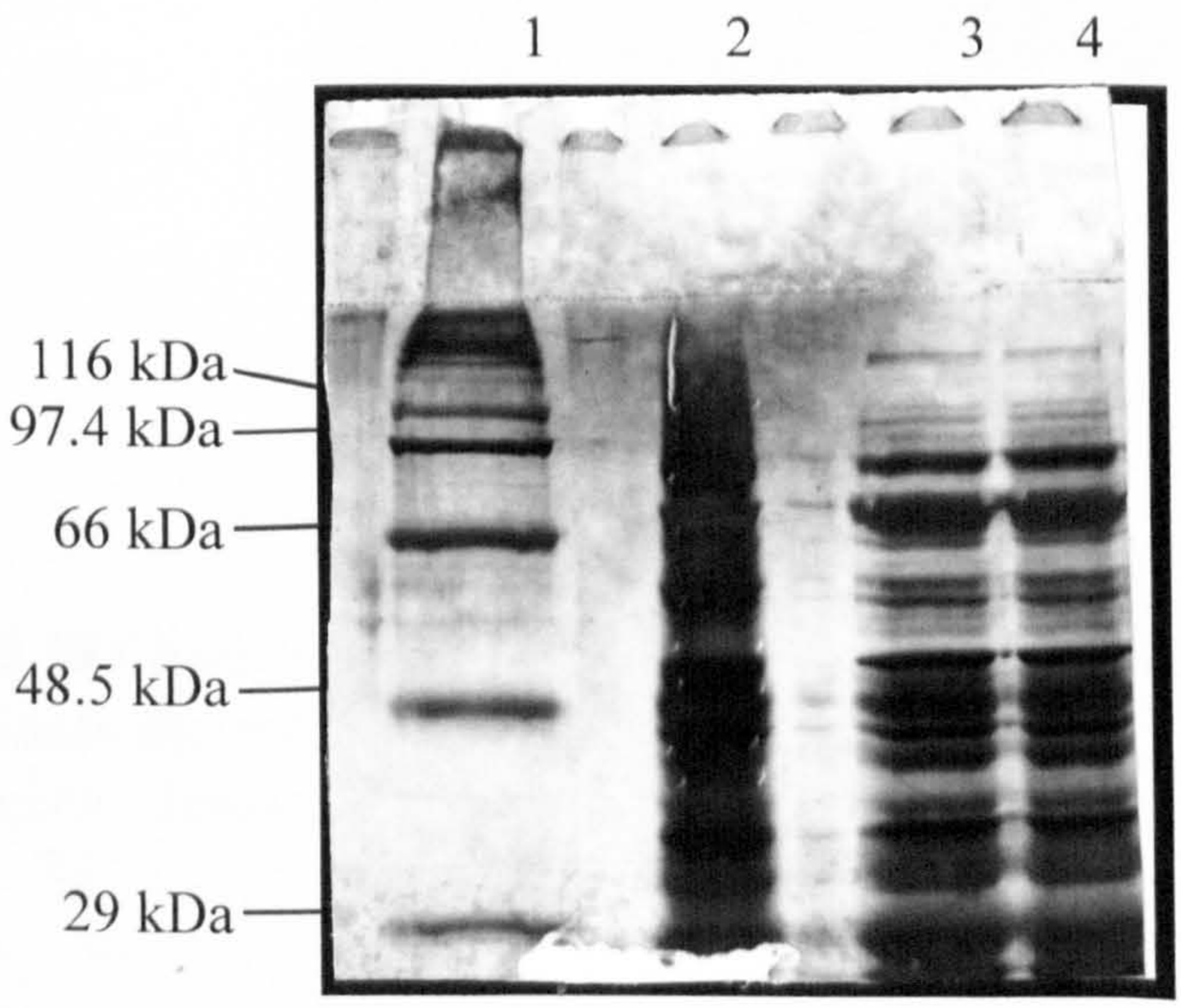


Figure 5.17 Silver stain of gel in figure 5.16.

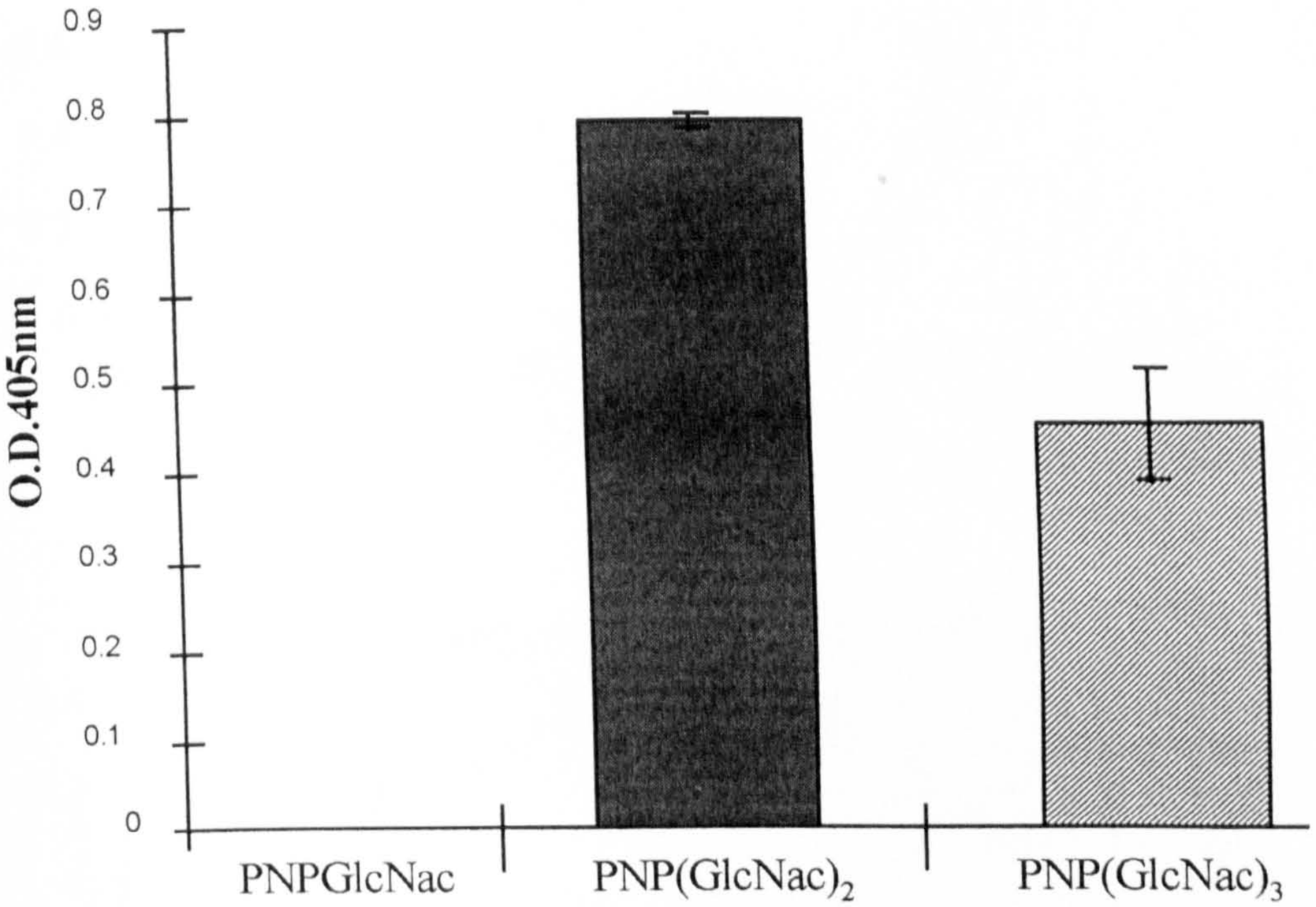


Figure 5.18 Characteristics of chitinase encoded by pMAT3
Exochitinase and endochitinase of whole cell lysate of *E.coli* DH5α(pMAT3), assayed with chromogenic chito-oligosaccharide derivatives.

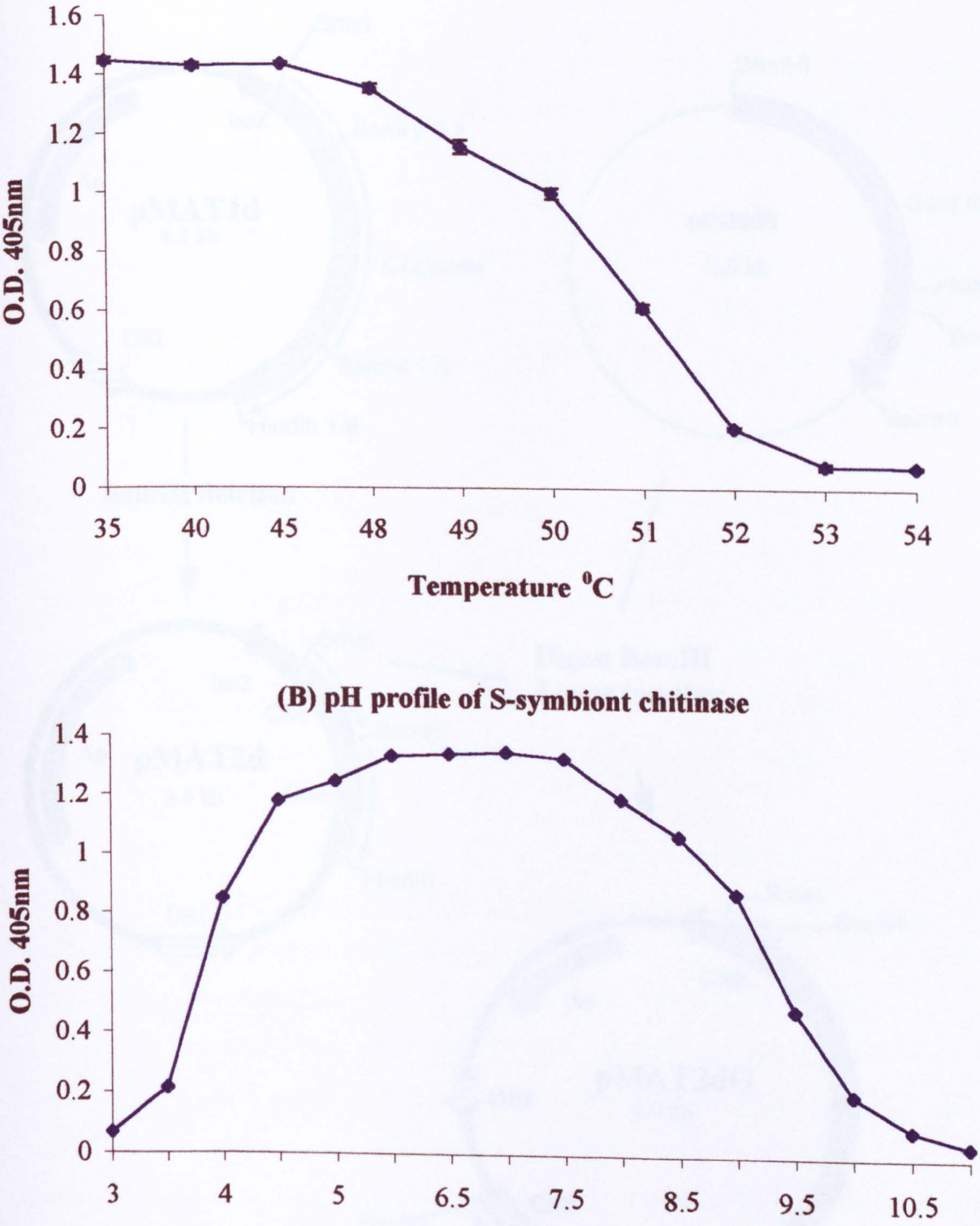


Figure 5.19 Characteristics of S-symbiont chitinase.
Chitinase activity was assayed using PNP(GlcNAc)2 and read at 405nm
(A) Displays the heat stability of chitinase encoded by pMAT3.
(B) Displays the activity of the chitinase encoded by pMAT3 at different pH's.
Chitinase activity detected using p-nitrophenol N,N'-diacetylchitobioside.

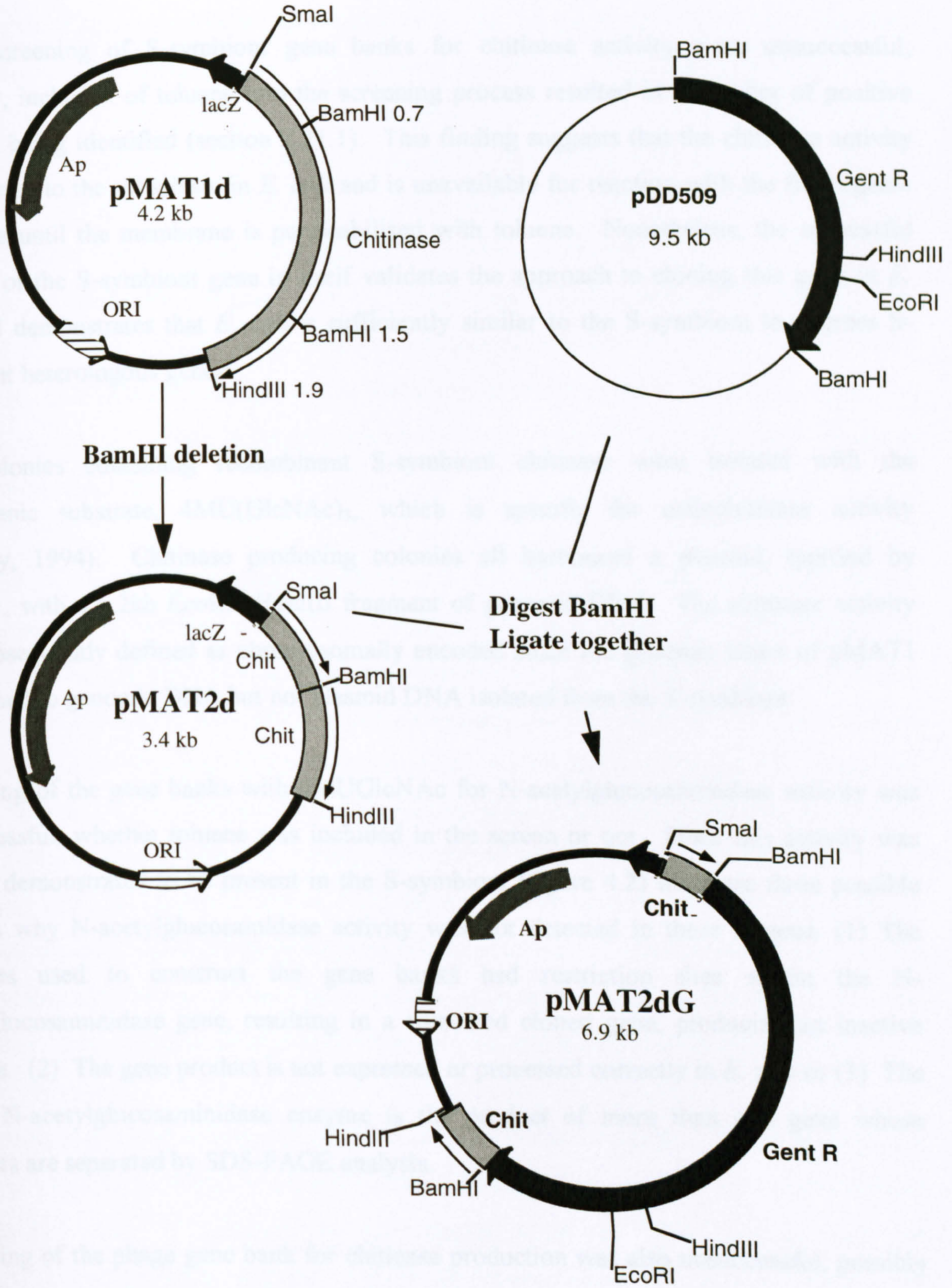


Figure 5.20 Construction of pMAT2dG

A construct for producing chitinase deletion mutants of the S-symbiont. Initially a 700 bp *Bam*HI deletion was made in pMAT1d by removal of a *Bam*HI fragment to form the plasmid pMAT2d. The 3.5kb gentamycin gene of pDD509 was then isolated as a *Bam*HI fragment and ligated into the unique *Bam*HI site of pMAT2d forming the plasmid pMAT2dG.

5.3 Discussion

Initial screening of S-symbiont gene banks for chitinase activity were unsuccessful; however, inclusion of toluene into the screening process resulted in a number of positive colonies being identified (section 2.21.1). This finding suggests that the chitinase activity is restricted to the cytoplasm in *E. coli* and is unavailable for reaction with the fluorogenic substrate until the membrane is permeabilised with toluene. Nonetheless, the successful cloning of the S-symbiont gene in itself validates the approach to cloning this gene in *E. coli* and demonstrates that *E. coli* is sufficiently similar to the S-symbiont to express S-symbiont heterologous genes.

The colonies containing recombinant S-symbiont chitinase were isolated with the fluorogenic substrate, 4MU$(GlcNAc)_3$, which is specific for endochitinase activity (Gooday, 1994). Chitinase producing colonies all harboured a plasmid, typified by pMAT1, with a 5.2kb *Eco*RI/ *Hin*dIII fragment of genomic DNA. The chitinase activity was subsequently defined as chromosomally encoded since the genomic insert of pMAT1 hybridised to genomic DNA but not plasmid DNA isolated from the S-symbiont.

Screening of the gene banks with 4MUGlcNAc for N-acetylglucosaminidase activity was unsuccessful, whether toluene was included in the screen or not. Since this activity was clearly demonstrated to be present in the S-symbiont (figure 4.2) there are three possible reasons why N-acetylglucosamidase activity was not detected in these screens. (1) The enzymes used to construct the gene banks had restriction sites within the N-acetylglucosaminidase gene, resulting in a truncated cloned gene, producing an inactive enzyme. (2) The gene product is not expressed or processed correctly in *E. coli* or (3) The active N-acetylglucosaminidase enzyme is the product of more than one gene whose products are separated by SDS-PAGE analysis.

Screening of the phage gene bank for chitinase production was also unsuccessful, possibly because the bacteria had insufficient time to produce the enzyme in detectable quantities before they were lysed by the phage. Phage libraries have been used previously by Robbins *et al.* (1988) to isolate a chitinase gene from *Streptomyces plicatus.* However, they noted that the signal produced using a fluorescent substrate was extremely weak and extended incubation was needed to detect it.

The analysis of chitinase in cell preparations of *E. coli* (pMAT1) by SDS-PAGE, together with subsequent renaturation of the enzyme and analysis by chitinase substrate overlay, revealed four bands of chitinase activity corresponding to species with molecular weights of 50kDa, 47kDa, 43kDa and 39kDa. The source organism displayed additional bands of chitinase activity of sizes 60kDa 75kDa, 105kDa and 130kDa. Multiple forms of chitinases are a common feature of bacterial chitinolytic systems (Berger and Reynolds, 1958; Roberts and Cabib, 1982; Watanabe *et al.*, 1990a; Fuji and Miyashita, 1991; Romaguera, 1992 and Shiro *et al.*, 1996), being produced either by expression of a number of chitinase genes, e.g. four ORFs encoding putative chitinases have been found in *Aeromonas* sp. No 10S-24 (Shiro *et al.*, 1996), or by post translational protease cleavage events, or indeed a combination of the two.

Deletion analysis and subcloning identified a 2.4kb fragment of genomic DNA at the *Hin*dIII end of the insert of pMAT1 as the sequence that encodes the chitinase gene(s). Sequence analysis of pMAT1b revealed the presence of a putative ORF, in part encoding a protein with homology to various other chitinases, which extended into the pUC18 polylinker. Therefore, despite encoding a protein with chitinase activity, it was concluded that the S-symbiont chitinase gene had been truncated by cloning using the enzyme *Hin*dIII. The missing DNA sequence was subsequently cloned and ligated to pMAT1a to form the plasmid pMAT3 containing the entire chitinase gene, designated *chi*A. Truncated chitinase genes that produce active products have been demonstrated for other bacterial systems; for example, deletion analysis of *Bacillus circulans* chitinase A1 revealed that though portions of the C terminus could be deleted without destroying activity (Watanabe *et al.*, 1990).

SDS-PAGE analysis of cell extracts of *E. coli* (pMAT3) revealed that pMAT3 encoded the full range of chitinase forms displayed by the wild-type S-symbiont. This result indicates that the full range of chitinase activity detected following SDS-PAGE analysis are produced by a single gene. Further, SDS-PAGE analysis using cell extracts boiled in the presence and absence of mercaptoethanol revealed that boiling caused a loss of all chitinase bands except one of 75 kDa and that the presence of mercaptoethanol during boiling had no effect. The largest band with chitinase activity without boiling is approximately 130kDa, which could indicate the formation of homodimers or formation of a heterodimer with a

second protein of size approximately 55kDa. This association must be due to strong electrostatic interactions between the two peptides since the association is not denatured by SDS in the SDS-PAGE analysis. The absence of any response to mercaptoethanol also suggest that disulphide bonds are not needed for the activity of the S-symbiont chitinase. The additional protein in a heterodimer could be a processed chitinases, for example, the chitinase form of size 50kDa, or it could be another protein encoded by the pMAT3 insert, although the former explanation is favoured. The symbiont chitinase was rapidly denatured at temperatures above 48°C as assayed using $PNP(GlcNAc)_2$ however as described above a single band of activity was still present when cell extracts were boiled for five minutes and then analysed by SDS-PAGE, this may be due to the much higher sensitivity of the fluorescent substrate detecting residual activity after boiling the cell extracts. Further characterisation of the S-symbiont chitinase showed that the S-symbiont displays more exochitinase than endochitinase activity since its activity against $PNP(GlcNAc)_2$ is greater than its activity against $PNP(GlcNAc)_3$ as shown in figure 5.18. This result is difficult to explain in that section 4 shows that the S-symbiont isolated from *G.m. morsitans* displayed more endochitinase activity than exochitinase assayed with the same substrates. In addition when detecting the chitinase activity following SDS-PAGE analysis of either S-symbiont or *E. coli* (pMAT3) cell extracts, $4MU(GlcNAc)_3$ give a better signal than $4MU(GlcNAc)_2$. Perhaps there is an additional endochitinase activity not encoded by the pMAT3 insert.

The chitinase encoded by the pMAT3 insert was further characterised with repect to pH activity profile and displays a broad pH optimum between pH 5.5 and 7.5 indistinguishable from the S-symbiont.

Finally attempts to produce chitinase mutants using the construct pMAT2dG were unsuccessful, this may be due to the previously noted inherent difficulty in transforming these bacteria in general.

Section 6

Sequence analysis of the chitinase gene <u>chiA</u> of the S-symbiont of <u>G. m. morsitans</u>

6.1 Introduction

Chapter four describes the identification and characterisation of chitinase activity in the S-symbiont isolated from *G. m. morsitans.* The organism displayed constitutive expression of chitobiase, exochitinase and endochitinase activity. Chapter five recorded the cloning of a gene *chiA* from this organism encoding a chitinase that displayed exochitinase and endochitinase activity. This chapter reports the sequencing and sequence analysis of *chiA.*

6.2 Results

6.2.1 Subcloning of pMAT1 for sequencing

The *Sal*I /*Hin*dIII subclone, pMAT1, described in section 5.2.7.2 contained the smallest piece of chromosomal symbiont DNA that was cloned and that encoded chitinolytic activity. The recovered fragment was then further subcloned into small overlapping pieces of DNA that could be easily sequenced on both strands using the M13 forward and reverse sequencing primers, designed to be complementary to sequences at either end of the multiple cloning site (MCS) of the pUC18 cloning vector.

6.2.1.1 Construction of pMAT1.3 and pMAT1.6

The plasmid pMAT1b was digested to completion with *Bam*HI and the resultant fragments separated by electrophoresis in an agarose gel. The DNA fragments of size 900bp and 700bp were purified and ligated, individually, to pUC18 that had previously been digested with the same enzyme and treated with CIP. The ligation mixes were dialysed and used to transform competent *E. coli* DH5α. Plasmids extracted from the resultant transformants were digested with *Bam*HI and the reaction products from each digest were analysed by gel electrophoresis and compared with a *Bam*HI digest of pMAT1b. Individual digests yielded inserts of 900bp and 700bp which aligned perfectly with the *Bam*HI fragments of pMAT1. The subclones were named pMAT1.3 and pMAT1.6 for the plasmid recombinants carrying the 900bp and 700bp inserts, respectively. The inserts were sequenced on both strands (figure 6.1).

6.2.1.2 Cloning of the 550bp BamHI/HindIII fragment pMAT1.8.

Plasmid pMAT1b was digested with *Hin*dIII and *Bam*HI and the fragments separated by agarose gel electrophoresis. The fragment of size 550bp was purified and ligated to pUC18 that had previously been digested *Bam*HI/*Hin*dIII. The ligation mix was used to transform competent *E. coli* DH5α and plasmids extracted from the resultant transformants were digested with *Bam*HI and *Hin*dIII to confirm that the correct piece of DNA had been cloned. The subclone was named pMAT 1.8 and its insert was sequenced on both strands (figure 6.1).

6.2.1.3 Cloning of the 700bp SalI/HindIII fragment pMAT1.7

Plasmid pMAT1b was digested to completion with *Sal*I and *Hin*dIII and the fragments were separated as described above. The fragment of size 700bp was purified and ligated to pUC18 that had been cut with the same enzymes. The ligation products were used to transform *E. coli* DH5α. Plasmid preparations isolated from the transformants were analysed by restriction mapping to confirm recovery of the desired fragment. Each contained a 700bp insert that aligned with the 700bp fragment of a *Sal*I/*Hin*dIII pMAT1b digest. The subclone was named pMAT1.7 and the insert was subsequently sequenced on both strands (figure 6.1).

6.2.1.4 Cloning of the 800bp SalI/SmaI fragment pMAT1.1

Plasmid pMAT1b was digested to completion with *Sal*I and *Sma*I, the DNA fragments were separated by electrophoresis and the fragment of size 800bp was purified and ligated to pUC18 that had also been digested with the same enzymes. The ligation mix was dialysed and used to transform competent *E. coli* DH5α. Plasmids isolated from the transformants produced were analysed by restriction digestion with *Sal*I and *Sma*I and found to carry a fragment of size 800bp that aligned perfectly with pMAT1b digested with *Sal*I/*Sma*I. The subclone was named pMAT1.1 and the insert was sequenced from both ends (figure 6.1).

6.2.1.5 Cloning of the 600bp BamHI/SmaI fragment pMAT 1.2

The *Bam*HI/*Sma*I 600bp fragment of pMAT1b was cloned as an *Eco*R1/*Bam*HI fragment by digestion of pMAT1b with *Bam*HI and *Eco*RI. *Eco*RI digests pMAT1b within the multiple cloning site of pUC18 at the *Sma*I end of the insert in pMAT1b. The 600bp band was purified, following restriction and electrophoresis and subsequently ligated into pUC18 that had been digested with the corresponding enzymes. The ligation products were used to transform *E. coli* DH5α and transformants were selected on nutrient agar containing ampicillin 100μgml^{-1}. Plasmids isolated from these transformants were digested with *Bam*HI and *Eco*RI and analysed on an agarose gel alongside an identical pMAT1b digest. Both lanes produced an identical 600bp band. The subclone was named pMAT1.2 and sequenced on both strands (figure 6.1).

6.2.1.6 Cloning of the 500bp SalI/BamHI fragment pMAT1.5

Plasmid pMAT1b was digested with *Bam*HI and *Sal*I to produce fragments of size 500bp and 200bp. The 500bp fragment was recovered by electro-elution after agarose gel electrophoresis and ligated to pUC18 that had previously been digested with the corresponding enzymes. The products of the ligation reaction were used to transform *E. coli* DH5α and transformants selected as above. Plasmids isolated from these transformants were digested with *Sal*I/*Bam*HI and analysed by gel electrophoresis alongside identical digests of pMAT1b. Both digests yielded a DNA fragment of 500bp. The subclone was named pMAT1.5 and the insert was sequenced on both strands (figure 6.1).

6.2.1.7 Cloning of the 1600bp SalI fragment pMAT1.4

pMAT1b was digested to completion with *Sal*I to produce fragments of size 3.8kb and 1.6kb. The fragment of size 1.6kb was electro-eluted after electrophoresis and ligated into pUC18 digested with *Sal*I. Plasmids extracted from transformants selected on ampicillin-containing agar plates (100μg.ml^{-1}) were digested with *Sal*I and analysed by gel electrophoresis as above. The presence of a band at 1.6kb confirmed that the correct subclone had been produced. The subclone was named pMAT1.4 and the insert was sequenced from both ends using the M13 primers complementary to sequences at the ends of the MCS of pUC18 (figure 6.1).

6.2.2 Additional sequencing

In addition to the subclones prepared above, the subclone pMAT1c (which was constructed as described in section 5.2.7.4) was also sequenced using the reverse sequencing primer which corresponds to pUC18 sequences at the *Eco*RI end of the MCS of pUC18. An oligonucleotide sequencing primer 7003 was also designed to sequence one strand of DNA at the *Sal*I end of the pMAT1b insert (figure 6.1).

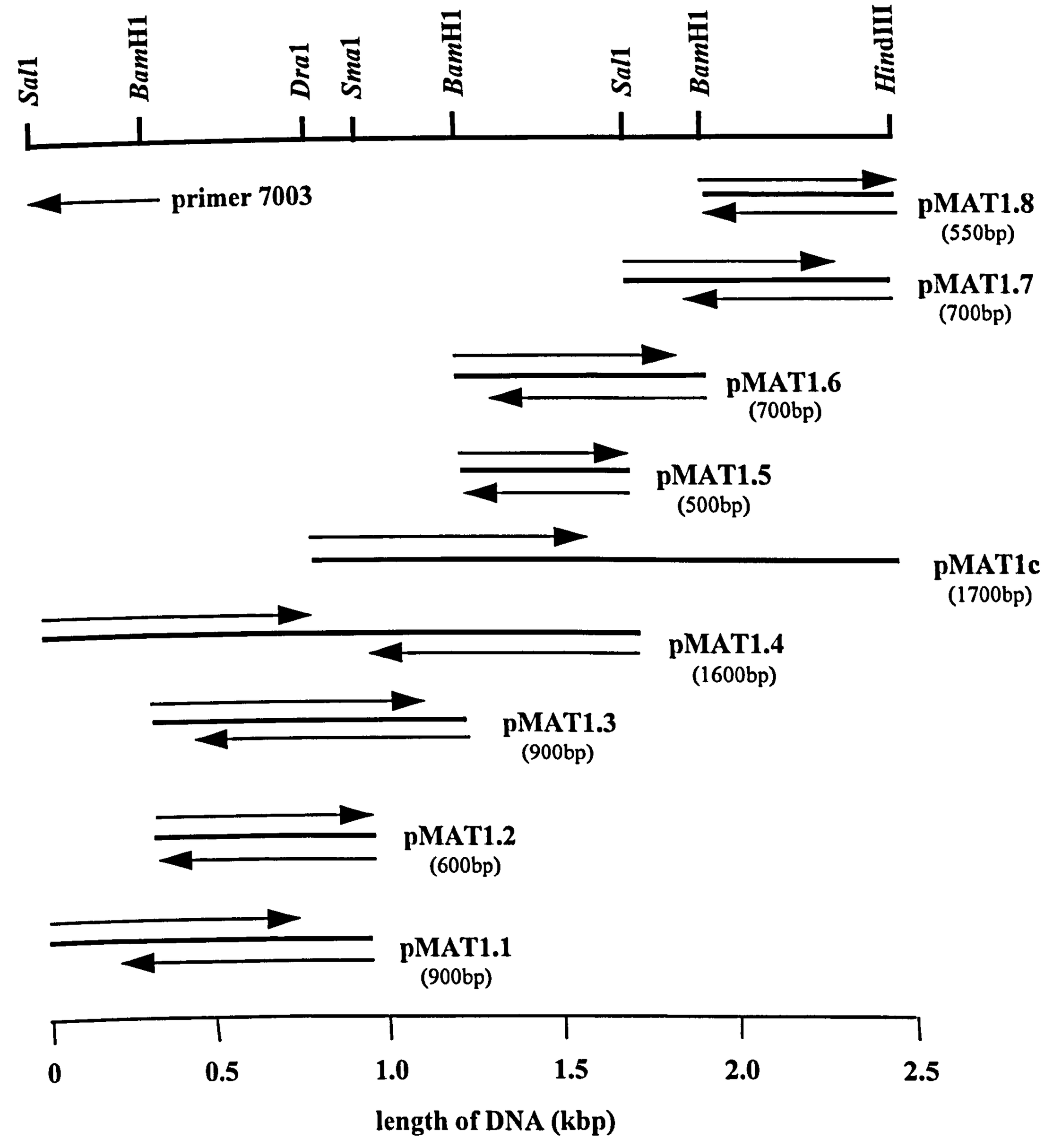


Figure 6.1 The sequencing strategy used to sequence pMAT1b.
Eight subclones were produced from the parent clone pMAT1b (as described in section 6.1) using the cloning vector pUC18. These subclones were sequenced on both strands using primers designed from M13 sequences flanking the MCS of pUC18. Additional sequences were also obtained from sequencing the pMAT1c subclone in the reverse direction and from the *Sal*I end of pMAT1b using the primer 7003 (as described in section 6.2.2). Arrows indicate the positions of the various sequencing runs and bold lines indicate the positions of the various subclones.

6.2.3 Reconstruction of entire sequence of pMAT1b insert.

The nucleotide sequences derived from sequencing the subclones pMAT1.1-1.8, pMAT1c and the sequence produced using the primer 7003 were imported into the DNA sequence analysis program "Genejockey" Biosoft Cambs. Forward and reverse sequences of each subclone were aligned. The corrected overlapping sequences were then aligned to each other and progressively joined together to make a contig of the entire pMAT1b insert.

6.2.4 Sequencing of pMAT2 containing 900bp *Bam*H1 insert.

The insert of plasmid pMAT2 constructed in section 5.2.10 was initially sequenced from both ends using oligonucleotide primers designed from M13 sequences flanking the MCS of pUC18. Two oligonucleotide sequencing primers were then designed from an internal 20 nucleotide sequence GATGAGCCAGTCTGTCTACC at the center of the pMAT2 subclone.

primer 1 :- 5'- GATGAGCCAGTCTGTCTACC -3',

primer 2 :- 5'- GGTAGACAGACTGGCTCATC -3'

pMAT 122 was then sequenced on both strands in opposite directions using the above primers (section 2.16). pMAT122 (as described in section 5.2.8.4) was sequenced so that an overlap was produced between the sequence already generated for pMAT1b and the adjacent DNA (figure 5.12). The sequences were then aligned and a contig produced between the sequence of pMAT1b and the adjacent DNA (pMAT2).

6.2.5 Sequence analysis of S-symbiont *chiA*

Sequence analysis of pMAT1b revealed the presence of a large incomplete ORF of size 1626 nucleotides. The ligation of the *Hin*dIII/*Bam*HI downstream fragment (pMAT2) to pMAT1a to create pMAT3 extended this ORF by a further 459 nucleotides (figure 5.12). The complete ORF encodes a predicted protein (S-symbiont Chi*A*) that shows similarity to a number of chitinases belonging to family 18 of the glycosyl hydrolases. The S-symbiont *chiA* has a theoretical molecular weight of 76 kDa and a theoretical pI of 4.78. Biophysical properties of S-symbiont *chi*A, including alpha and beta regions, hydrophilicity plot etc, are

shown in appendix A. The GC content of S-symbiont *chi*A is 50.6% and the codon preference of *chi*A has the values NNA, 15%; NNU,30%; NNC,32% and NNG,23%. Further sequence analysis using PSORT prediction of protein localisation sites version 6.4 revealed that the predicted protein does not possess an N-terminal signal sequence for export (appendix B).

6.2.6 Comparison of S-symbiont chiA with other chitinases

Protein sequence data bases were searched for sequences similar to that of S-symbiont *chi*A using BLASTP (Altschul *et al.*, 1990) via the National Center for Biotechnology Information's BLAST WWW Server. Post processing was provided by BEAUTY, Human Genome Center, Baylor College of Medicine (Worley *et al.*, 1995). The results revealed that the sequence of S-symbiont *chi*A was similar to a host of other chitinases of prokaryotic, eukaryotic and viral origin. The highest scores were found for *Aeromonas caviae* chitinase, *Enterobacter agglomerans* chitinase, *Serratia marcescens chi*A, *Alteromonas chi*85, the chitinase from Autographa californica nuclear polyhedrosis virus, the chitinase from *Paramecium bursaria,* Chlorella virus and *Bacillus circulans chi*A1 in order of descending scores. Alignment of S-symbiont *chi*A with *Aeromonas caviae* chitinase was performed using the "ALIGN optimal global alignment of 2 sequences with no short cuts" program at EERIE Nimes France using their WWW network server, and the alignment is shown in figure 6.3. Multiple alignment of the four highest scoring sequences was performed using CLUSTALW and the alignment is displayed in figure 6.4. The multiple alignment reveals that S-symbiont *chi*A has a number of blocks of homology with the other chitinases; notably, homology is high in the vicinity of the glutamic acid residue at position 249 and the aspartate residue at position 322, of the S-symbiont chitinase. The equivalent residues in *Serratia marcescens ChiA* and *Bacillus circulans ChiA1* have been shown to be essential for catalysis (Watanabe *et al*, 1993; Perrakis *et al*, 1994). A further multiple alignment, performed with the addition of the chitinase A1 sequence from the Gram positive bacterium *Bacillus circulans* was used to produce a comparison matrix of these chitinases (figure 6.5). This alignment was further used to produce a phylogenetic ancestral tree for these chitinases using Lasergene software DNAstar West Ealing (figure 6.6). A further computer search, performed using the C-terminal 90 amino acids of S-symbiont ChiA, revealed that a portion of the C-terminus was homologous to putative chitin binding domains in other bacterial chitinases, as well as to a

domain in a *Bacillus circulans* cellulase. These homologous sequences were used to produce a multiple alignment using ClustalW (figure 6.7) and sequence pair distances, deduced using Lasergene (DNASTAR West Ealing, London) (figure 6.5).

6.2.7 Prediction of the promoter regions and ribosome binding site for S-symbiont *chi*A

The putative -35, -10 promoter regions, and the putative ribosome binding site (RBS) are highlighted in figure 6.2. The promoter regions and RBS are similar to *E. coli* consensus sequences, the spacing between the promoter sequences being the optimal 16 bases (Gold and Stormo, 1987; Hoopes and McClure, 1987). The promoter region occurs 608 bp from the end of the pMAT1b inserted fragment and, therefore, it is unlikely that the expression of S-symbiont *chi*A is influenced by external factors, such as a hybrid promoter. Thirty six base pairs distal to the termination codon is a nine base pair perfect inverted repeat sequence separated by a five base pair loop and followed by a T rich region, which would be a U rich region in mRNA. These sequences probably function in transcriptional termination.

```
   1                                                        CTGCAGTCGAC
  12 AAGTTCAAACATATTATTTTCCGTGCTATCGTCCAATACGGTATCGG
  59 TAATTTATGCGTGGGCGCCGGCGCCACCCGTGAAGCTATTGGTAAGG
 106 TCTATGAGCTTGCAAAAGAAAAAGGCATTCAAACCCTGGGACGGGTG
 153 TCGGAACAAGCGCGAGAAAGCACGTCGCTGTCTGAACATTGTGATGA
 200 ATGCATTTTCATTTCTGACCCGATGGCATCCTGGCAAGTGCGGGATC
 247 CAGAGGGAAACTCCTACATGGTTTATCTGGATACCGACAAGGCGGTC
 294 ATACAGCGTACCGGCGAGTTTCTGGCTTTTGGCGGAGGTCAGATCAC
 341 ATCACGCTTAAAGAACTTGAAGAGGCTGAGCGCTCAGGTATCAAACC
 388 AACCGTTTATCCTGATTTTACAACGTGCGCCCGCCGTGTCACCGCAC
 435 GCAGGCAGAGTGACCCCCATAGCGATGTAACGCCCGTCCGTATCGCC
 482 TATAGGGACTTGAAACCGCCGCCTTGTCGTTAACCTGCATAATGGCA
 529 TTACCGTGATGTAACGTTCACAGGTCAAGCAGTGCCTTTTTCAAGTC
 576 TGGTTTATTAAATCACAATACAACACCGGTGCATTGATAAAGTTTAT     -35
 623 ATTTTTAATAAAATACCCTCCATATCAAATCAAAGTAAGTTTTATTC     -10
 670 ATAACGAGGAAGGTAGCTCATTCAAAAGGTGAACGTGGTTAGTTTTT
 717 AAAATTACATATCTCTTAAATCATAATAACTTTGTAAGGTAACTAAC     SD
     Met Ser Ser Gln Leu Ile Gln Lys Asp Gln Tyr Ser      12
 764 ATG AGC AGT CAG TTA ATT CAG AAA GAC CAA TAT AGC
     Asp Glu Ser Tyr Gln Tyr Asp Gly Phe Asp Pro Lys      24
 801 GAT GAG TCA TAT CAG TAT GAT GGT TTT GAC CCT AAG
     Thr Asn Asp Ser Ala Tyr Ser Tyr Thr Ser Ala Arg      36
 837 ACC AAT GAT TCG GCC TAC AGC TAT ACC TCT GCC CGG
     Val Met Lys Arg Val Tyr Asn Lys Tyr Asp Thr Lys      48
 873 GTG ATG AAA CGC GTT TAT AAT AAA TAC GAC ACC AAG
     Asn Lys Pro Lys Val Phe Gly Tyr Tyr Thr Asp Trp      60
 909 AAC AAA CCA AAA GTG TTT GGC TAT TAT ACC GAC TGG
     Gly Gln Tyr Asp Gly Arg Ala Leu Ser Ser Pro Pro      72
 945 GGA CAG TAC GAT GGC AGA GCA TTA TCA TCG CCA CCC
     Ser Gly Ser Val Asp Val Gly Ser Arg Gly Arg Gly      84
 981 TCC GGT AGC GTT GAT GTC GGG AGT CGT GGC CGC GGG
     Val Asp Phe Ser Gln Leu Ser Pro Thr Ala Tyr Asp      96
1017 GTC GAT TTC TCG CAA TTA TCC CCG ACC GCC TAT GAT
     Lys Ile Ile Phe Gly Phe Thr Gly Ile Val Gly Asp     108
1053 AAA ATT ATT TTC GGA TTT ACC GGT ATT GTC GGC GAT
     Lys Gly Ala Asn Gln Tyr Lys Ile Glu Gln Ala Ala     120
1089 AAA GGT GCC AAT CAG TAT AAA ATC GAG CAG GCC GCG
     Ala Trp Thr Gly Lys Lys Gln Tyr Glu Met Thr Ile     132
1125 GCG TGG ACC GGG AAA AAG CAA TAT GAA ATG ACC ATT
     Leu Asp Pro Trp Gly Asp Cys Gln Ala Tyr Phe Asn     144
1161 CTG GAT CCC TGG GGT GAT TGT CAG GCT TAC TTC AAC
     Asn Gly Phe Ser Ser Tyr Lys Asp Phe Gly Phe Gly     156
1197 AAC GGT TTT TCA AGT TAT AAA GAT TTT GGC TTC GGT
     Pro Gly Thr Thr Tyr Asn Gly Gly Ser Gln Glu Asp     168
1233 CCC GGC ACT ACA TAC AAT GGC GGA TCG CAG GAA GAT
     Cys Phe Lys Glu Ser His Pro Asn Leu Gln Gly Val     180
1269 TGC TTC AAA GAA AGT CAC CCC AAT CTG CAA GGG GTG
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Figure 6.2 DNA sequence of chitinase gene of pMAT3 and deduced amino acid sequence. Amino acid residues are numbered on the right hand side of the figure from the start codon. Nucleotides are numbered on the left hand side of the figure from *Sal*I site of pMAT1b. Potential -35, -10 and Shine Delgarno sequences are portrayed in bold and underlined.

(S-symbiont chitinase accession number Y11391)

```
      Leu Gly Ala Leu Leu Ala Leu Lys Lys Lys Ala Ala     192
1305  CTG GGC GCC CTA CTG GCT CTG AAG AAA AAA GCG GCG
      Leu Ala Gly His Asp Leu Ala Leu Ser Phe Ser Val     204
1341  CTG GCA GGG CAT GAT CTT GCG CTT TCT TTC AGC GTC
      Gly Gly Trp Thr Met Ser Glu Ile Phe His Glu Met     216
1377  GGC GGC TGG ACG ATG AGC GAA ATC TTC CAT GAA ATG
      Val Lys Ser Asp Gln Ala Ile Asn Thr Phe Val Ser     228
1413  GTC AAA AGT GAC CAG GCG ATC AAC ACT TTC GTC AGC
      Ser Ile Val Asp Phe Phe Gln Arg Phe Pro Ser Phe     240
1449  AGC ATC GTT GAT TTC TTT CAA CGC TTC CCC AGC TTC
      Ser Glu Ile Asp Ile Asp Trp Glu Tyr Pro Asn Ala     252
1485  TCT GAA ATC GAT ATT GAC TGG GAA TAT CCG AAT GCC
      Ala Gly Ala Gly Asn Pro His Gly Pro Glu Asp Gly     264
1521  GCA GGT GCC GGT AAC CCC CAT GGC CCT GAA GAC GGT
      Ala Asn Tyr Gln Lys Leu Ile Ala Ala Leu Arg Gln     276
1557  GCG AAT TAC CAA AAA TTG ATT GCT GCT CTG CGT CAG
      Ala Phe Asn Ser His Asn Arg Gln Asp Ile Lys Ile     288
1593  GCG TTT AAT AGC CAT AAC CGG CAA GAT ATC AAA ATC
      Ser Ile Ala Ser Ser Ala Asn Val Asp Val Leu Gln     300
1629  AGT ATC GCT TCG TCC GCC AAT GTC GAC GTC CTG CAA
      His Ser Asn Ile Lys Gly Leu Leu Ala Ala Gly Leu     312
1665  CAT TCC AAT ATC AAA GGG CTG TTA GCT GCC GGG TTA
      Tyr Gly Ile Asn Val Met Thr Tyr Asp Phe Phe Gly     324
1701  TAC GGT ATC AAC GTT ATG ACC TAT GAC TTC TTT GGT
      Thr Pro Trp His Glu Gly Leu Thr Asn His Thr Asn     336
1737  ACG CCG TGG CAC GAA GGA TTG ACG AAT CAC ACC AAT
      Leu Tyr Lys Thr Glu His Ser Ser Tyr Ser Leu Glu     348
1773  CTG TAT AAA ACT GAG CAT TCC AGT TAT AGC CTG GAA
      Glu Ala Val Thr Tyr Leu Leu Glu Gln Gly Val Asp     360
1809  GAG GCG GTG ACC TAT CTG CTA GAA CAG GGG GTG GAT
      Pro Asp Val Ile Asn Val Gly Tyr Ala Gly Tyr Ser     372
1845  CCG GAC GTC ATT AAT GTG GGC TAT GCT GGT TAT TCC
      Arg Ser Ala Lys Gly Ala Glu Ile Ser Ser Phe Ser     384
1881  CGT AGC GCC AAA GGT GCC GAA ATC AGC TCT TTC TCG
      Pro Leu Lys Gly Thr Tyr Glu Gly Asn Asp Thr Thr     396
1917  CCG CTG AAG GGC ACC TAT GAA GGT AAT GAC ACC ACC
      Val Gly Thr Phe Glu Ser Gly Cys Val Glu Trp Tyr     408
1953  GTC GGC ACG TTC GAG TCA GGT TGT GTT GAA TGG TAT
      Asp Val Leu Tyr Asn Tyr Leu Asp Leu Glu Asn Lys     420
1989  GAC GTT CTT TAT AAC TAT CTC GAT TTG GAA AAT AAA
      Ser Gly Arg Asn Gly Tyr Gln Val Tyr Thr Asp Asp     432
2025  TCA GGT CGC AAT GGC TAC CAG GTT TAT ACC GAT GAT
      Val Ala Cys Ala Asp Tyr Leu Tyr Ser Pro Thr Ala     444
2061  GTC GCC TGT GCC GAC TAT CTG TAT AGC CCG ACG GCG
      Lys Val Phe His Ser Ile Asp Thr Pro Arg Ser Val     456
2097  AAG GTT TTC CAT TCC ATT GAT ACA CCG CGT AGC GTC
      Arg Glu Lys Ala Arg Tyr Val Ile Glu Lys Gly Leu     468
2133  AGG GAA AAA GCC CGC TAT GTG ATT GAA AAA GGT CTT
```

Figure 6.2 Continuation of Sequence of chitinase gene of pMAT3 and deduced amino acid sequence. **(S-symbiont chitinase accession number Y11391)**

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      Gly Gly Ile Phe Thr Trp Thr Ile Asp Tyr Asp Asn     480
2169  GGC GGT ATT TTT ACC TGG ACC ATC GAT TAC GAC AAT
      Gly Leu Leu Val Asn Ala Ala Arg Glu Gly Leu Gly     492
2205  GGC TTG CTG GTC AAC GCA GCG CGT GAA GGG TTG GGC
      Cys Pro Ile Val Asp Lys Val Ile Asp Met Ser Pro     504
2241  TGT CCG ATT GTT GAT AAG GTC ATC GAT ATG AGT CCA
      Phe Tyr Phe Lys Gly Ile Asn Ile Thr Gly Glu Asp     516
2277  TTC TAT TTC AAG GGG ATT AAT ATC ACT GGT GAA GAT
      Glu Gly Lys Pro Asp Glu Pro Asn Thr Pro Asp Lys     528
2313  GAA GGC AAG CCG GAT GAA CCG AAT ACC CCT GAC AAA
      Pro Ala Ala Ala Pro Val Ala Lys Val Glu Ile Lys     540
2349  CCA GCG GCA GCA CCG GTC GCC AAG GTT GAA ATT AAA   HindIII site
      Ala Phe Ala Gly Ser Ser Leu Leu Phe Cys Gly Gln     552
2385  GCT TTC GCG GGC AGT TCG TTA CTG TTC TGT GGT CAG
      Gln Ser Val Asn Ala Ala Cys Tyr Glu Trp Ser Ala     564
2421  CAG TCC GTC AAT GCC GCC TGC TAT GAA TGG TCC GCC
      Thr Gln Gly Ala Val Ile Ala Ala Pro His Ala Glu     576
2457  ACC CAG GGG GCC GTC ATC GCC GCG CCG CAT GCC GAA
      Gln Thr Ala Val Val Leu Pro Asn Val Ser Val Asp     588
2493  CAG ACT GCG GTA GTG CTG CCT AAC GTC AGC GTG GAT
      Thr Leu Ile Ser Ile Thr Leu Ala Val Thr Asn Glu     600
2529  ACC CTC ATA TCC ATA ACC CTC GCC GTG ACG AAC GAA
      Ser Gly Glu Arg Ala Thr Ala Val Phe Ala Leu Thr     612
2565  AGC GGT GAG CGG GCA ACC GCT GTT TTC GCG TTA ACG
      Val Val Pro Lys Asp Asp Thr Asp Glu Gln Pro Glu     624
2601  GTT GTT CCA AAG GAT GAC ACG GAC GAG CAG CCG GAA
      Thr Pro Asp Glu Pro Glu Thr Pro Ser Gln Tyr Gln     636
2637  ACG CCG GAT GAA CCG GAA ACC CCT TCG CAA TAT CAG
      Gln Trp Ile Ala Thr Gln Ile Tyr Thr Glu Gly Asn     648
2673  CAG TGG ATA GCC ACT CAG ATC TAC ACC GAA GGC AAC
      Leu Val Ser His Lys Gly Val Asp Tyr Arg Ala Asn     660
2709  CTG GTT TCC CAT AAA GGC GTC GAT TAT CGG GCC AAC
      His Trp Ser Gln Gly Asp Glu Pro Val Cys Leu Pro     672
2745  CAT TGG AGT CAG GGG GAT GAG CCA GTC TGT CTA CCA
      Pro Pro Asp Ser Met Val Ser Arg Gly Arg Cys Trp     684
2781  CCA CCG GAC AGT ATG GTT TCC CGT GGT CGG TGC TGG
      Leu Pro Asp Pro Ile Ala Leu Tyr Arg Phe Gly Stop     695
2817  TTG CCT GAT CCC ATT GCG TTA TAC CGA TTC GGG TGA
2853  ACGTCGTCACCCACGTTGACTCTTATCGATGCATCCACCCGCCGTCA   inv
2900  GACACGGCGGGTTTGTTACATCTGGTCTTTGTGATCCTATTGACAGC   repeat
2947  CTTCGCCGATTGCCTGACCCTGACCCGAGCGCCCTGGCATCATTGCG    36bp
2994  TTCTAGTGTATCGATTCCGCCGCAGGCGCGCATCGCGCTATTTGGCC    from
3041  ATTTCACCGATCAATATTGCGGTATGGACCCTTAGCGGCGCGTTGGC    stop
3088  TATCGCCTGCGTTCGCCGCTGGAAAGCGCTTACCACGTTAATGAGCG    codon
3135  GGTCCGTCATGTTACTGTTCGGCGTCGCCGCCATTCTGGCAGGCGCC
3182  AATGCGGGATCC
```

Figure 6.2 Continued DNA sequence of S-symbiont chitinase gene and deduced amino acid sequence. The pMAT1b clone ends at the *Hin*dIII enzyme site at nucleotide position 2385bp which is in bold and underlined. A 9bp perfect inverted repeat is portrayed in bold and underlined 36 base pairs distal to translation termination signal which is followed by a T rich run which would be a U rich run in mRNA.

34.9% identity in 324 aa overlap; score: 504

```
253 KGNFGNLMALKQ----AHPDLKILPSVGGWTLSDPFYFLGDKTKR-DTFVASVKEFLQTW 308
    .: .: :.:::.    :  :: .  :::::::.:. :. .  . .  .:::.:. .:.: .
178 QGVLGALLALKKKAALAGHDLALSFSVGGWTMSEIFHEMVKSDQAINTFVSSIVDFFQRF 238

309 KFFDGVDIDWEFPGGQGA-NPSLGGPNDGATYVVLMKELRAMLDELEAETGRQYELTSAI 368
      :. .:::::.:.. :: ::   ::.:::.:  :.  ::  ..   ... .. ... :
239 PSFSEIDIDWEYPNAAGAGNPH--GPEDGANYQKLIAALRQAFN---SHNRQDIKISIAS 294

369 SAGGDKIAKVDYQAAQQY-MDHIFLMSYDFSGAFDLTNLAHQTNLFGSSWDPATKYTTDK 428
    ::. : . . . ..     .  : .:.::: :.    .:...:::. .      ..:. ..
295 SANVDVLQHSNIKGLLAAGLYGINVMTYDFFGTPWHEGLTNHTNLYKTE---HSSYSLEE 352

429 GVKALLGQGVTPGKIVVGAAMYGRGWTGVKNYQAGNPFTGTATG--PVSGTWENGVVDYR 487
    .:  :: ::: :  : :: : :.:.  :..  .. .:. ::  :   . ::.:.: :..
353 AVTYLLEQGVDPDVINVGYAGYSRSAKGAE-ISSFSPLKGTYEGNDTTVGTFESGCVEWY 412

488 DIV------NNRMGAGWEQGY-DESAEAPYVFKASSGDLITFDNDRSVKTKGQYVLANQL 541
    :..      .:. : .  : : :. : : :... ..  . ..:. :::. :..::. . :
413 DVLYNYLDLENKSGRNGYQVYTDDVACADYLYSPTAKVFHSIDTPRSVREKARYVIEKGL 473

542 GGLFAWEIDADNGDILNAMHEGLG 566
    ::.:.: :: ::: ..:: .::::
474 GGIFTWTIDYDNGLLVNAAREGLG 498
```

29.8% identity in 151 aa overlap; score: 179

```
659 KAPQPNLP--PVVSVPASATVEAGKQVSIKATASDPNGDALSYQWTVPAGLSATGLDSAT 717
    :  .:: :  :... :.. ..:    :.    .. . .:  :.:..  :   ..  .
519 KPDEPNTPDKPAAAPVAKVEIKAFAGSSL-LFCGQQSVNAACYEWSATQGAVIAAPHAEQ 578

718 LVVTGSNVTSDTVYDLTLVVTDGALD-ATAVTRLIVKPAS-TGGGCEASD-PDA-ANHPA 774
     .:.  ::. ::. ..:::.::. . . :::::  : : : . :    :. : :.. ...
579 TAVVLPNVSVDTLISITLAVTNESGERATAVFALTVVPKDDTDEQPETPDEPETPSQYQQ 637

775 WSAGTVYNTNDKVSHKQLVWQAKYWTQGNEP 805
    : :  .:. .. :::: . ..:..:.::.::
638 WIATQIYTEGNLVSHKGVDYRANHWSQGDEP 669
```

38.0% identity in 50 aa overlap; score: 128

```
801 NEPSRTADQWKLVSQVQLGWDAGVVYNGGDVTSHNGRKWKAQYWTKGDEP 851
    .:  .: :. .  :: :  : :  .:. :...::.:  ..:..:..:::: 
620 DEQPETPDEPETPSQYQQ-WIATQIYTEGNLVSHKGVDYRANHWSQGDEP 668
```

26.4% identity in 72 aa overlap; score: 90

```
165 VEWGVYGRKFTVDKIPAQNLTHILYGFTPICGGNGINDSLKEISGSFEALQRSCAGREDF 224
    :. :  ::    ...      .:..::: : : .: :.   : ....        .:....
 76 VDVGSRGRGVDFSQLSPTAYDKIIFGFTGIVGDKGANQYKIEQAAAW-------TGKKQY 128

225 KVSIHDPWAAIQ 236
    ...: :::.  :
129 EMTILDPWGDCQ 140
```

Figure 6.3 Alignment of deduced amino acid sequences of *Aeromonas caviae* chitinase and S-symbiont chitinase. Residues identical in *A.caviae* chitinase and S-symbiont chitinase are shown with colons. Conservative changes are indicated with dots. The S-symbiont chitinase is depicted as the lower line in each alignment. The alignment was generated using the ALIGN application at EERIE Nimes France using their network server.

22.9% identity in 109 aa overlap; score: 60

```
600 ALTYSWKQVSGPQASLLDVTQAKARVVLDAVSSDINLVFELTVTDDQGLSAKDQVVVT-- 660
    :  : :. ..:   .   . :.   ::: :: :  . . :.::...:  :     ..:
557 AACYEWSATQGAVIAAPHAEQTA--VVLPNVSVDTLISITLAVTNESGERATAVFALTVV 614

661 ---NKAPQPNLPPVVSVPASATVEAGKQVSIKATASDPNG-DALSYQWT 702
       .   ::. :    .:..     . :.  ...  . .: :  . .:.
615 PKDDTDEQPETPDEPETPSQYQQWIATQIYTEGNLVSHKGVDYRANHWS 663
```

34.4% identity in 32 aa overlap; score: 60

```
145 LKENNKPYANKSGKVVGAYYVEWGVY-GRKFT 238
    .:.  . : .:.   : .::..:: : :: ..
 38 MKRVYNKYDTKNKPKVFGYYTDWGQYDGRALS  69
```

22.7% identity in 88 aa overlap; score: 52

```
751 VKPASTGGGCEASDPDAANHPAWSAGTVYNTNDKVSHKQLVWQAKYWTQGNEPSRTA-DQ 809
    :  .: : : . :. . . .     : .  ..:: ...  . ::  ::  ..   : :
 76 VDVGSRGRGVDFSQLSPTAYDKIIFGFTGIVGDKGANQYKIEQAAAWTGKKQYEMTILDP 135

810 W-KLVSQVQLGWDAGVVYNGGDVTSHNG 839
    :    .  . :...   .. :  :..::
136 WGDCQAYFNNGFSSYKDFGFGPGTTYNG 163
```

43.8% identity in 16 aa overlap; score: 48

```
814 SQVQLGWDAGVVYNGG 829
    :  ..:.  :..::::
149 SYKDFGFGPGTTYNGG 164
```

27.8% identity in 36 aa overlap; score: 47

```
749 LIVKPASTGGGCEASDPDAANHPAWSAGTVYNTNDK 784
    :.:. :  : ::   :       : .  :   . .:.
482 LLVNAAREGLGCPIVDKVIDMSPFYFKGINITGEDE 517
```

19.8% identity in 111 aa overlap; score: 44

```
382 MDHIFLMSYDFSGAFDLTNLAHQTNLFGSSWDPATKYTTDKGVKALLGQGVTPGKIVVGA 441
    : . :. .    :  . ::: ..:.  . : . :. : ..::   . .    :    .
318 MTYDFFGTPWHEGLTNHTNL-YKTEHSSYSLEEAVTYLLEQGVDPDVINVGYAGYSRSAK 376

442 AMYGRGWTGVKNYQAGNPFTGTATGPVSGTWENGVVDYRDIVNNRMGAGWE 492
    .   ... .:.   ::  :  .       : . . .: :. :.    :..
377 GAEISSFSPLKGTYEGNDTTVGTFESGCVEWYDVLYNYLDLENKSGRNGYQ 427
```

Figure 6.3 continued

```
1 S.marcescens    MRKFNKPLLALLIGS TLCSAAQAAAPGKPT IAWGNTKFAIVEVDQ AATAYNNLVKV-KNA  59
2 E.agglomerans   MRKFNKPLLALLIGS TLCSAAQAAAPGKPS IASGPTKFAIVEVDQ QATAYNNLVKL-KTA  59
3 A.caviae        MLSPKLSLLALLVGG LCTTSAFAAAPAKPT IGSGPTKFAIVEVNQ AASAYNQLVTVHKDG  60
4 S-symbiont      ------MSSQLIQKD QYSDESYQYDGFDPK TN--DSAYSYTSARV MKRVYNKYDTK--NK  50

1 S.marcescens    ADVSVSWNLWNGDAG TTAKILLNGKEAWSG PSTGSSGTANFKVNK G-GRYQMQVALCNAD 118
2 E.agglomerans   ADVSISWDLWSGDAG TTAKVLLDGKEVWSG ASTGTSGTANFKVNK G-GRYQMQVALCNAD 118
3 A.caviae        APVSVTWNLWSGDVG QTAKVLLDVKEVWSG PAS-AAGTANFKVTK G-GRYQMQVALCNAD 118
4 S-symbiont      PKVFGYYTDWG---- -----QYDGRALSS- PPS---GSVDVGS-R GRGVDFSQLSPTAYD  96

1 S.marcescens    GCTASDATEIVVADT DGSHLAPLKEPLLEK NKPYKQNSGKVVGSY FVEWGVYGRNFTVDK 178
2 E.agglomerans   GCTASDVTQIVVADT DGSHLAPLKEPLLEN NKPYKQDSGKVVGSY FVEWGVYGRNFTVDK 178
3 A.caviae        GCTLSDKKELMVADT DGSHLAPLNAPLKEN NKPYANKSGKVVGAY YVEWGVYGRKFTVDK 178
4 S-symbiont      KIIFG--FTGIVGD- KGANQYKIEQAAAWT GK--KQ----YEMTI LDPWGDCQAYFNNG- 146

1 S.marcescens    IPAQNLTHLLYGFIP ICGGNGINDSLKEIE GSFQALQRSCQGRED FKVSIHDPFAALQKA 238
2 E.agglomerans   IPAQNLTHLLYGFIP VCGGDGINDSLKGVE GSFQALQRSCQGRED FKVSIHDPFAAVQKG 238
3 A.caviae        IPAQNLTHILYGFTP ICGGNGINDSLKEIS GSFEALQRSCAGRED FKVSIHDPWAAIQMS 238
4 S-symbiont      FS----SYKDFGFGP GTTYNG-------- GS----QEDC----- FKESHP---NLQGV 180
```

Figure 6.4 The putative amino acid sequence alignments of the chitinases from 1, *Serratia marcescens chiA; 2, Enterobacter agglomerans; 3, Aeromonas caviae* and 4, S-symbiont (isolated from *Glossina morsitans morsitans*). Residues identical to those of the S-symbiont chitinase are blocked and shaded. Dashes represent gaps or shorter molecules at the N or C terminus. The alignment was generated using CLUSTAL

```
1 S.marcescens   QKGVTAWDDPYKGNF GQLMALKQAHPDLKI LPSIGGWTLSDPFFF MG-DKVKRDRFVGSV 297
2 E.agglomerans QKGVTAWDDPYKGNF GQLMALKQARPDLKI LPSIGGWTLSDPFFF MG-DKVKRDRFVGSV 297
3 A.caviae      QGNLSAWDEPYKGNF GNLMALKQAHPDLKI LPSVGGWTLSDPFYF LG-DKTKRDTFVASV 297
4 S-symbiont    LGALLALKK------ ---KAALAGH-DLAL SFSVGGWTMSEIFHE MVKSDQAINTFVSSI 231

                                *
1 S.marcescens   KEFLQTWKFFDGVDI DWEFPGGKGANPNLG SPQDGETYVLLMKEL RAMLDQLSAETGRKY 357
2 E.agglomerans KEFLQTWKFFDGVDI DWEFPGGGGANPKLG NAQDGATYVQLMKDL RAMLDQLSAETGRKY 357
3 A.caviae      KEFLQTWKFFDGVDI DWEFPGGQGANPSLG GPNDGATYVVLMKEL RAMLDELEAETGRQY 357
4 S-symbiont    VDFFQRFPSFSEIDI DWEYPNAAGAGNPHG -PEDGANYQKLIAAL RQAFNSHNRQD---I 287

                                *
1 S.marcescens   ELTSAISAGKDKIDK VAYN-VAQNSMDHIF LMSYDFYGAFDLKNL GHQTALNARPGSRHR 416
2 E.agglomerans ELTSAISAGKDKIDK VDYN-TAQNSMDHIF LMSYDFYGAFDLKNL GHQTALKARPGNRHG 416
3 A.caviae      ELTSAISAGGDKIAK VDYQ-AAQQYMDHIF LMSYDFSGAFDLTNL AHQTNLFGSSWDPAT 416
4 S-symbiont    KISIASSANVDVLQH SNIKGLLAAGLYGIN VMTYDFFGTPWHEGL TNHTNLYK---TEHS 344

1 S.marcescens   LHHGER-RQCAAGQG VKPGKVVVGIAMYGR GWTGVNGYQNNIPFT GTATG--PVKGTWKN 473
2 E.agglomerans LYHGER-RQCVAGQG VKPGKIVVGAAKYGR GWTGVSGYQNNNPFT GTATG--PVKGTWEN 473
3 A.caviae      KYTTDKGVKALLGQG VTPGKIVVGAAMYGR GWTGVKNYQAGNPFT GTATG--PVSGTWEN 474
4 S-symbiont    SYSLEEAVTYLLEQG VDPDVINVGYAGYSR SAKGAEIS-SFSPLK GTYEGNDTTVGTFES 403
```

Figure 6.4 continued The conserved Glu- 315 and Asp-391 residues located at the active site of *Serratia marcescens* ChiA (Perrakis *et al*, 1994) are shown by asterixes.

```
1 S.marcescens   GIVDYRQIAGQFMSG E-------WQYTYDA TAEAPYVFKPSTGDL ITFDDARSVQAKGKY 526
2 E.agglomerans  GIVDYRQIANEFISD E-------WQYSYDA TAEAPYVFKPSTGDL ITFDDPRSVQAKGKY 526
3 A.caviae       GVVDYRDIVNNRMGA G-------WEQGYDE SAEAPYVFKASSGDL ITFDNDRSVKTKGQY 527
4 S-symbiont     GCVEWYDVLYNYLDL ENKSGRNGYQVYTDD VACADYLYSPTAKVF HSIDTPRSVREKARY 463

1 S.marcescens   VLDKQLGGLFSWEID ADNGDILNSMNASLG NSAGVQ--------- ---------------- 562
2 E.agglomerans  VLDKQLGGLFSWEID ADNGDILNNMNTSLG NSAGAQ--------- ---------------- 562
3 A.caviae       VLANQLGGLFAWEID ADNGDILNAMHEGLG HGEGTLPPANKPPVA NAGSDLSATGPAEVT 587
4 S-symbiont     VIEKGLGGIFTWTID YDNGLLVNAARDGLG CPIVDKVIDMSPFYF KG---INITGEDE-- 518

1 S.marcescens   ---------------- ---------------- ---------------- ---------------- 562
2 E.agglomerans  ---------------- ---------------- ---------------- ---------------- 562
3 A.caviae       LNGSASHDPENGALT YSWKQVSGPQASLLD VTQAKARVVLDAVSS DINLVFELTVTDDQG 647
4 S-symbiont     ----GKPDEPN----- ---------------- ---------------- ----------TPDK- 529

1 S.marcescens   ---------------- ---------------- ---------------- ---------------- 526
2 E.agglomerans  ---------------- ---------------- ---------------- ---------------- 526
3 A.caviae       LSAKDQVVVTNKAPQ PNLPPVVSVPASATV EAGKQVSIKATASDP NGDALSYQWTVPAGL 707
4 S-symbiont     ---------------- PAAAPVAKVEIKAFA GSSLLFCGQQS---- -VNAACYEWSATQGA 569
```

Figure 6.4 continued

```
1 S.marcescens   ---------------- ---------------- ---------------- ---------------- 526
2 E.agglomerans  ---------------- ---------------- ---------------- ---------------- 526
3 A.caviae       SATGLDSATLVVTGS NVTSDTVYDLTLVVT -DGALDATAVTRLIV KP-ASTGGGCEASD- 764
4 S-symbiont     VIAAPHAEQTAVVLP NVSVDTLISITLAVT NESGERATAVFALTV VPKDDTDEQPETPDE 629

1 S.marcescens   ---------------- ---------------- ---------------- ---------------- 526
2 E.agglomerans  ---------------- ---------------- ---------------- ---------------- 526
2 A.caviae       P-DAANHPAWSAGTV YNTNDKVSHKQLVWQ AKYWTQGNEPSRTAD QWKLVSQVQLGW-DA 822
4 S-symbiont     PETPSQYQQWIATQI YTEGNLVSHKGVDYR ANHWSQGDEPVCLPP PDSMVSRGRCWLPDP 689

1 S.marcesens    ---------------- ---------------- ---------------- 526
2 E.agglomerans  ---------------- ---------------- ---------------- 526
2 A.caviae       GVVYNGGDVTSHNGR KWKAQYWTKGDEPGK AAVWVDQGAASCN 867
4 S-symbiont     IALYRFG--------- ---------------- ---------------- 696
```

Figure 6.4 continued

Percent Similarity

Percent Divergence

	1	2	3	4	5	6	
1		35.9	21.9	19.4	70.1	70.5	1
2	48.3		18.2	16.8	42.3	42.7	2
3	70.8	71.8		16.5	20.5	21.0	3
4	73.7	74.8	75.3		17.1	16.7	4
5	27.9	47.2	67.3	72.2		90.4	5
6	27.7	47.5	66.6	73.6	9.6		6
	1	2	3	4	5	6	

A. caviae chitinase

Alteromonas chitinase

B. circulans chiA

S-Symbiont chiA

E. agglomerans chitinase

S.marcescens chiA

Figure 6.5 Comparison matrix of S-symbiont chiA with other chitinases showing sequence pair distances, using Clustal method (Pearson and Lipman, 1988)

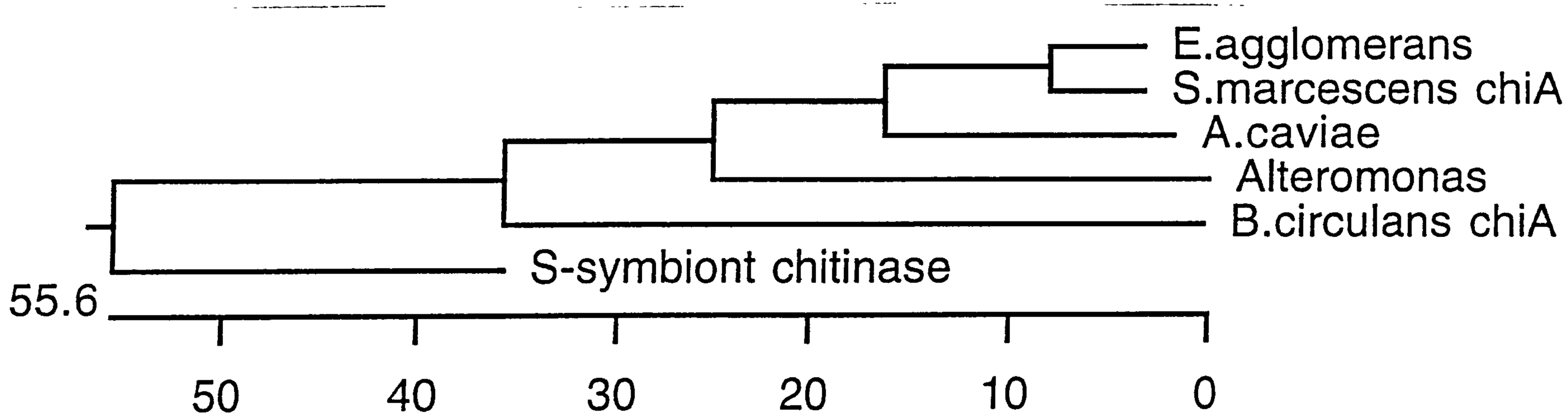


Figure 6.6 Phylogenetic ancestral tree for amino acid sequences of S-symbiont chiA and five other chitinases belonging to family 18 of the glycosyl hydrolases. The length of each pair represents the distance between sequence pairs. The scale beneath the tree measures the distance between sequences (Lasergene navigator, DNASTAR, West Ealing, London, UK.)

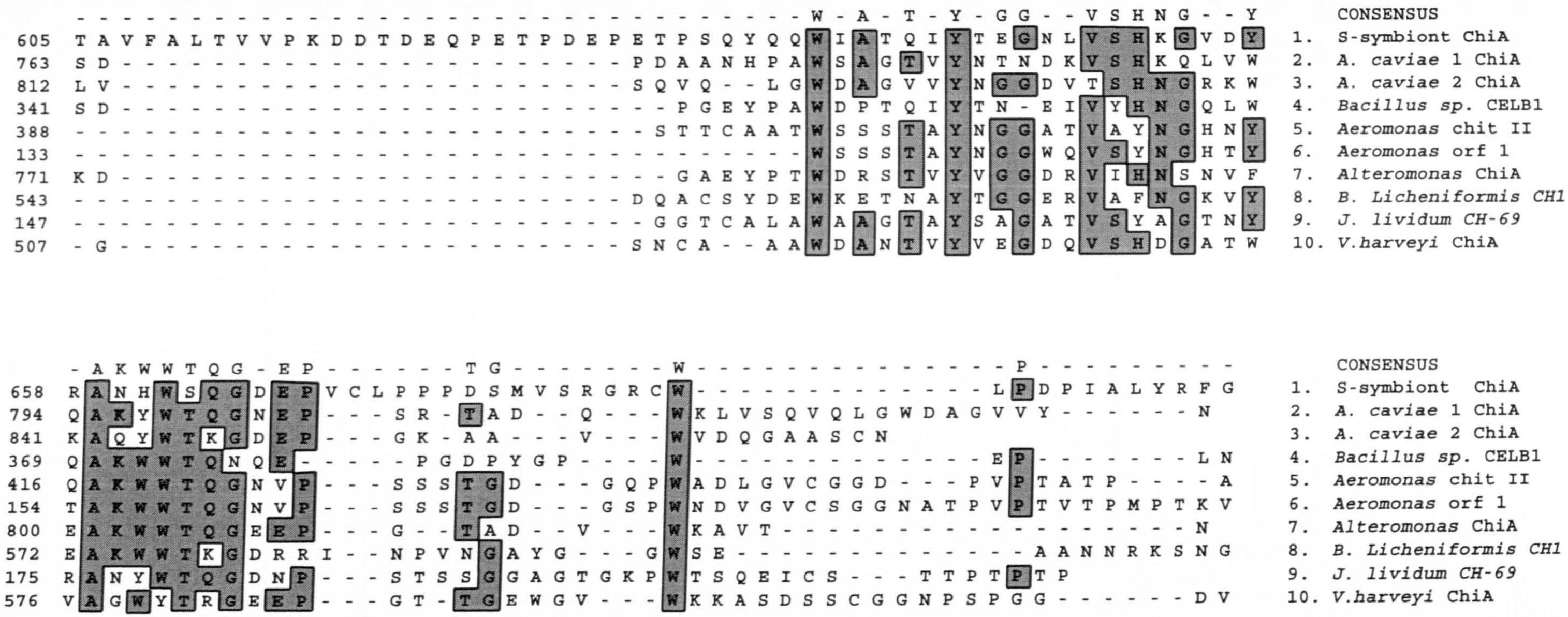


Figure 6.7 Alignment of the predicted C-terminal amino acid sequence of S-symbiont ChiA with the predicted amino acid sequences of :- **2** and **3,** C-terminal portions of ChiA from *Aeromonas caviae,* amino acids 763-828 and 812-866, respectively (Sitrit *et al.*, 1995); **4**. cellulase CELB1 from *Bacillus sp.,* aa's 341-390 (Sanchez-Torres *et al.*, 1996); **5**, chitinase II of *Aeromonas sp.*, aa's 388-453 (Ueda *et al.*, 1994); **6**, chitinase of ORF 1 from *Aeromonas* sp., aa's 133-198 (Shiro *et al.*, 1996); **7**, CHIA from *Alteromonas sp.* strain 0-7 aa's 771-821 (Tsujibo *et al.*, 1992); **8**, chitinase (CH1) from *Bacillus licheniformis,* aa's 543-604 (Acc No U71214); **9**, CHI-69 from *Janthinobacterium lividum,* aa's 147-212 (Gleave *et al.*, 1995), and **10**, CHIA from *Vibrio harveyi* aa's 507-613 (Acc No U81496) Amino acid residues present in five or more sequences are blocked and shaded.

6.3 Discussion

Sequence analysis revealed that the fragment of S-symbiont DNA cloned within pMAT3 contained a large open reading frame of size 2088 nucleotides, encoding a predicted protein of 695 amino acids, with a theoretical molecular mass of 76kDa and a pI of 4.78. The predicted size of the product agrees well with the band of chitinase activity of 75kDa following SDS-PAGE analysis and subsequent renaturation of boiled samples of *E. coli* DH5α (pMAT3).

The open reading frame is preceded by a putative Shine Delgarno sequence 6bp upstream from the ATG start codon. Further upstream sequences were found that show a high degree of similarity to typical *E. coli* σ70 promoter regions, with the -10 and -35 boxes separated by 16 nucleotides. The ORF was also followed by a stem loop structure 36bp distal to the stop codon, which probably functions in translation termination.

Comparison of the predicted amino acid sequence of S-symbiont ChiA with other chitinases revealed that it shares the greatest homology with ChiA from *Aeromonas cavaie* followed by *Enterobacter agglomerans* chitinase, *Alteromonas* ChiA and *Serratia marcescens* ChiA. These chitinases belong to family 18 of the glycosyl hydrolases and therefore S-symbiont ChiA is also a member of this family of chitinases. Homology with *A. caviae* was greatest in a 324bp overlap between residues 178-498 of S-symbiont ChiA and amino acids 253-566 of *A. caviae* ChiA which were 34.9% identical. This section of *A. caviae* ChiA has previously been shown to display high similarity to *Alteromonas* ChiA and *S. marcescens* ChiA (Tsujibo *et al.*, 1993; Sitrit *et al.*, 1995). In particular Asp391 and Glu315 residues of *S. marcescens* ChiA, suggested to be involved in the acid-base catalysis of chitin are conserved in all of these chitinases, including S-symbiont ChiA (Watanabe *et al*, 1992; Watanabe *et al*, 1993; Perrakis *et al*, 1994). The high homology displayed by these chitinases over this catalytic region suggests that all these enzymes share similar 3D structures, which has been demonstrated to consist of an α/β barrel fold for *S. marcescens* ChiA.

The S-symbiont chitinase displayed no homology with the fibronectin type III domains previously found in a number of different chitinases (Blaak *et al.*, 1993; Watanabe *et al.*,

1993) as well as various bacterial carbohydrases (Meinke *et al.*, 1991; Bork and Doolittle, 1992; Blaak *et al.*, 1993). In this respect S-symbiont chitinase is more similar to Chi69 from *J.lividum* (Gleave *et al.*, 1995), *S. thermoviolaceus* Chi40 (Tsujibo *et al.*, 1993) and ChiA from *S. marcescens* (Perrakis *et al.*, 1994) which also lack the fibronectin type III domains, indicating that these elements are not essential for chitinase activity. Their function remains unclear.

A computer search of the C-terminal 90 amino acids revealed that the C-terminus contained a section of sequence displaying homology to a number of chitinases including the C-terminal repeats of *A. caviae*, showing 29% and 30% identity, respectively, for repeat 1 and 2, as well as 32.5% identity with a cellulase from *Bacillus circulans.* The homology between the repeated regions in *A. caviae* and cellulases from *B. circulans* has been described previously by Sitrit *et al.* (1995). These authors suggested that the hydrophobic residues conserved in this region may provide a hydrophobic protein environment facilitating binding to high molecular weight substrates like chitin and cellulose. This particular domain was found in different positions within the chitinase genes from different organisms and was not found exclusively in the C-terminal, further illustrating the diverse arrangement of domains within chitinases.

Of particular interest was the region of S-symbiont ChiA immediately preceding this putative chitin binding domain, including residues 615-635. This section contained four aspartate residues, four glutamic acid residues and five proline residues producing a concentration of negatively charged residues. Clearly, the fact that S-symbiont *chiA* was initially cloned truncated and lacking its C-terminus shows that the C-terminal is not essential for activity and therefore must serve some other function. The fact that the truncated gene product could not associate with other protein(s) to produce a larger product suggests that the C terminus may be involved in the ability of S-symbiont ChiA to form dimers/ multimers; to date this is the first example of a bacterial chitinase that dimerises. Furthermore the presence of this sequence in S-symbiont ChiA and its absence in other chitinases suggests that this sequence may be involved in the ability of S-symbiont chitinase to form dimers/multimers

The sequence of S-symbiont chitinase also sheds light on the chitinase activity of various

subclones produced from the pMAT1b plasmid. The plasmid pMAT1c was cloned via the *Dra*I and *Hin*dIII sites of the insert within pMAT1b.

Since the *Dra*I enzyme site is found between the promoter and the ATG start codon of S-symbiont chitinase, *E. coli* transformed with pMAT1c would not be expected to produce an active product unless as an in-frame fusion with β-galactosidase. It is therefore not surprising that this subclone does not produce an active product. pMAT1d is a *Sma*I/*Hin*dIII derivative of pMAT1b lacking the first 100bp of the S-symbiont ChiA sequence and would not contain promoter sequences or a start codon and also would not be expected to produce a functional chitinase.

The putative amino acid sequence of S-symbiont ChiA lacks a classical N-terminal signal sequence. Dominant features of signal sequences typically consist of an N-terminus of five to six amino acids with a net positive charge, a hydrophobic core of about 12 highly hydrophobic residues and a C-terminus of six amino acids with neutral small side-chain amino acids at positions -1 and -3 often including a proline or glycine residue (Izard and Kendall, 1994). This type of sequence functions in increasing the efficiency of protein transport across membranes. Attempts to determine the N-terminal sequence via Edman sequencing (Hesswick, 1974) failed due to being N-terminally blocked. Therefore direct empirical evidence of the N-terminus is lacking, although the start codon of S-symbiont *chiA* is preceded by a reasonable Shine Delgarno sequence and is in keeping with the position of a promoter with strong homology to σ70 type promoters of *E. coli*. Furthermore, the sequence between the putative start codon and the -10 sequence of the promoter contains 8 stop codons including representatives in all three reading frames. In addition to this, no typical signal sequence is found in any reading frame upstream or downstream of this start codon within the pMAT1b insert and it is therefore highly likely that the S-symbiont ChiA does not contain a typical N-terminal signal sequence.

Attempts at cell fractionation of the S-symbionts were unsuccessful. However, zones of fluorescence were detected around colony lifts of S-symbionts when exposed to 4MU$(GlcNAc)_3$, whereas no fluorescence was detected when colonies of *E. coli* were treated similarly. These preliminary results suggest that the chitinase is restricted to the cytoplasm in *E. coli* but is exported or secreted in the S-symbiont. However leakage of chitinase by mechanical damage cannot be ruled out. If the chitinase is exported/secreted

by the S-symbiont the lack of a N-terminal signal sequence suggests it is likely to be exported via a Sec independent pathway. A number of proteins are exported via Sec independent processes, including chitinase B from *Serratia marcescens* (Brurberg *et al.*, 1995).

The chitinase locus of the S-symbiont has a GC content of 50%. This is consistent with the organism being placed within the Enterobacteriaceae although, as discussed, the sequence of the chitinase from the Gram positive bacterium *Bacillus circulans* showed greater similarity to chitinases from enterobacterial origin than the S-symbiont chitinase did. This may reflect the niche in which the S-symbiont is found, the chitinase having evolved to suit life within the tsetse fly. As yet free living variants of this bacterium have not been found.

Section Seven

Sequencing and sequence analysis of DNA upstream of S-symbiont (G. m. morsitans) chiA

7.1 Introduction

Many genes that have related functions can be cloned together and, of particular interest, is the knowledge that a number of bacterial chitinases, N-acetylglucosaminidases and chitinase-related regulatory genes have been cloned by virtue of their position close to other chitinase structural genes. The possibility of finding genes encoding for products such as permeases for the uptake of degradation products of the S-symbiont chitinase suggested that such an investigation was worthwhile. Since pMAT1 contained an additional 2.8kb of DNA upstream of the subclone pMAT1b, this was sequenced to search for interesting genes.

7.2 Results

7.2.1 Sequencing strategy

The DNA upstream of the chitinase gene was sequenced using a combination of subcloning and custom primer sequencing. Initially, the *Eco*RI/*Sph*I subclone pMAT1.9 was produced by digestion of pMAT1 with *Eco*RI/*Sph*I and ligation of the 1.3kb fragment into suitably digested pUC18. This subclone was subsequently sequenced from both ends of its insert using primers designed against the DNA flanking the multiple cloning site of pUC18. Custom primers (6761, 7000) were then designed against suitable sequences to extend the sequence. The subclone pMAT1a was additionally sequenced in the forward and reverse directions and extended with custom primers 7001 and 6774, respectively (figure 7.1). The two sequences produced by combining these overlapping contigs were linked via the sequence produced from primer 7002 used to sequence a portion of pMAT1 corresponding to the junction of clones pMAT1.9 and pMAT1. The sequence of the upstream region of DNA is listed in appendix C

7.2.2 Sequence analysis of upstream DNA

Analysis of the completed sequence identified a number of open reading frames for the entire sequence of the pMAT3 insert (figure 7.2). The largest open reading frame was that of the chitinase. A number of other putative open reading frames were identified. The open reading frames were translated into putative amino acid sequences and used to search the protein sequence data bases for similar sequences using BLASTP

(Altschul *et. al.*, 1990) via the National Center for Biotechnology Information's BLAST WWW Server. Post processing was provided by BEAUTY, Human Genome Center, Baylor College of Medicine (Worley *et. al.*, 1995). None of the deduced sequences were significantly similar to any proteins in the data bases.

Figure 7.1 Strategy for sequencing upstream of S-symbiont *chiA*

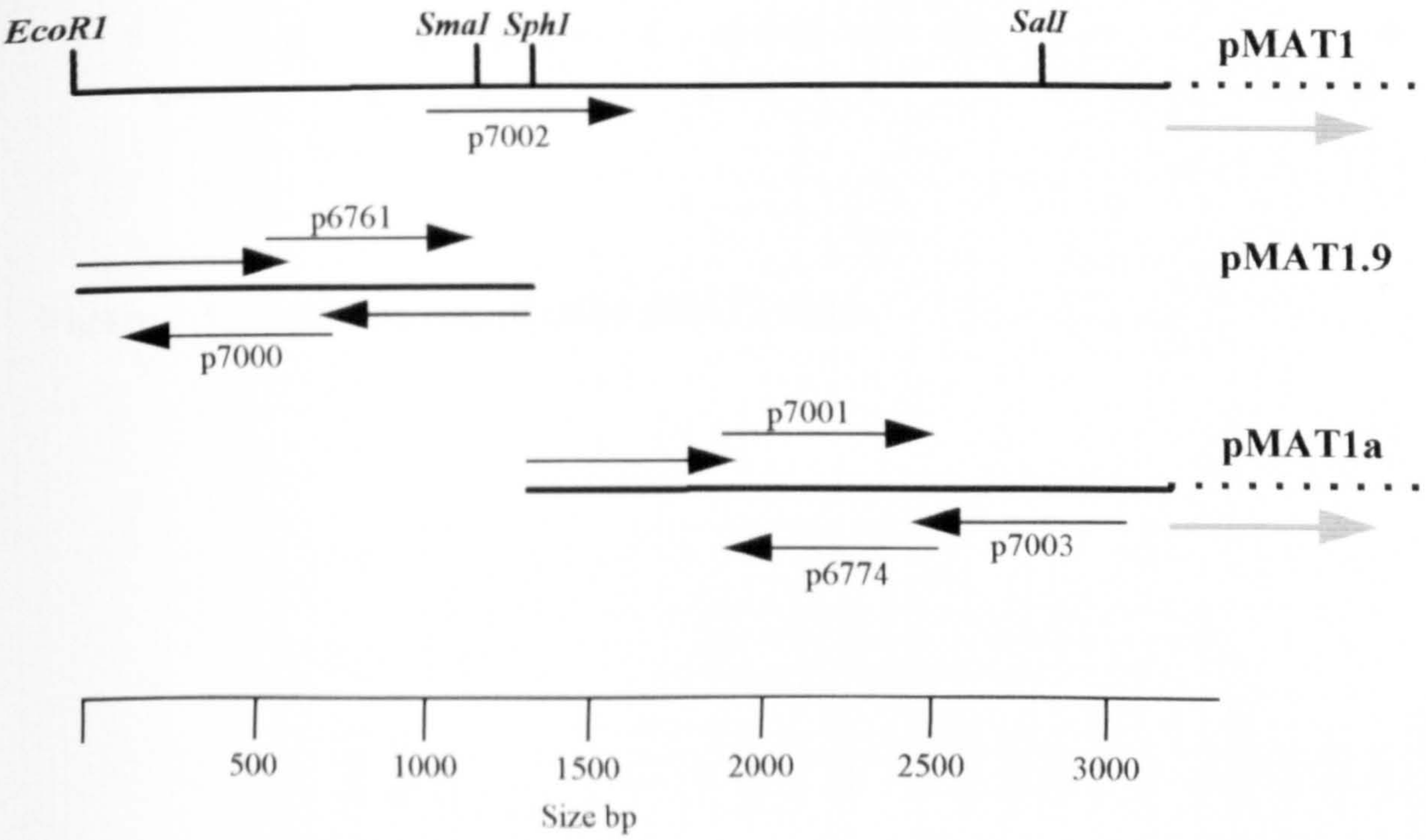


Figure 7.1 Strategy for sequencing the pMAT1.9 and pMAT1a inserts. Sequences were initially produced via oligonucleotides designed against sequences flanking the multiple cloning site of pUC18. These sequences were then extended via custom primers; 7001,7003,6774 and 6761, 7000. Contigs produced were then joined with the sequence produced from oligonucleotide 7002 Black arrows denote individual sequence runs, shaded arrows indicate the position of S-symbiont *chi*A gene.

Figure 7.2 Open reading frames found in all six reading frames of pMAT3 including upstream of *chiA* (6490bp).

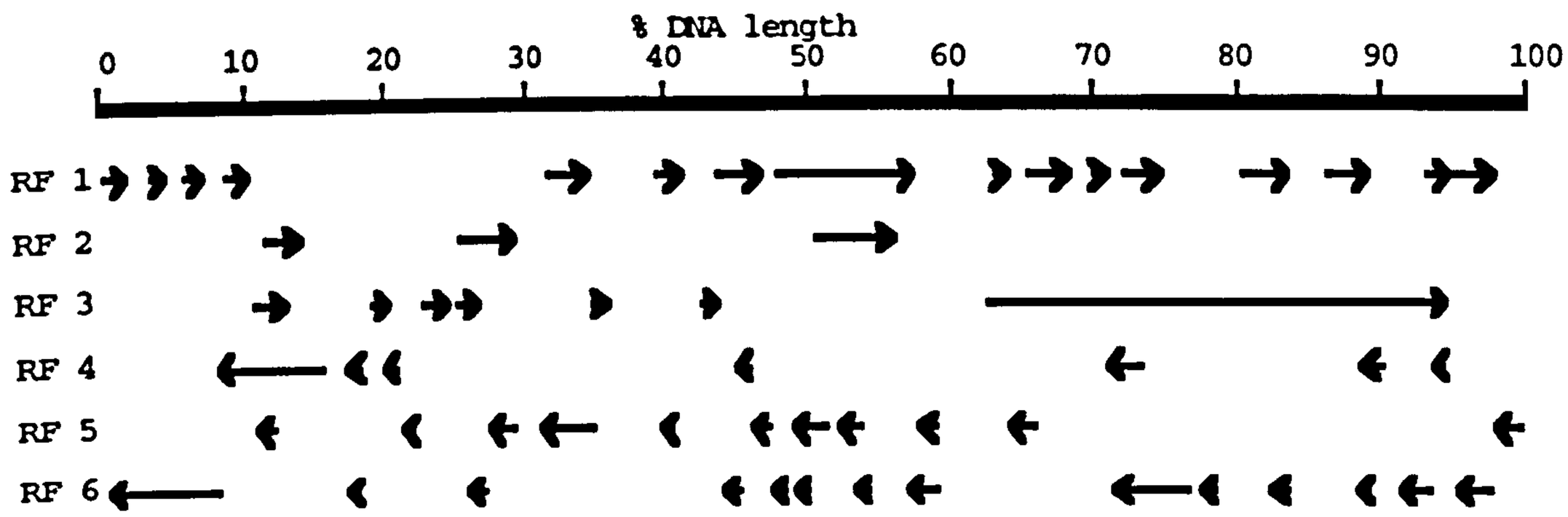
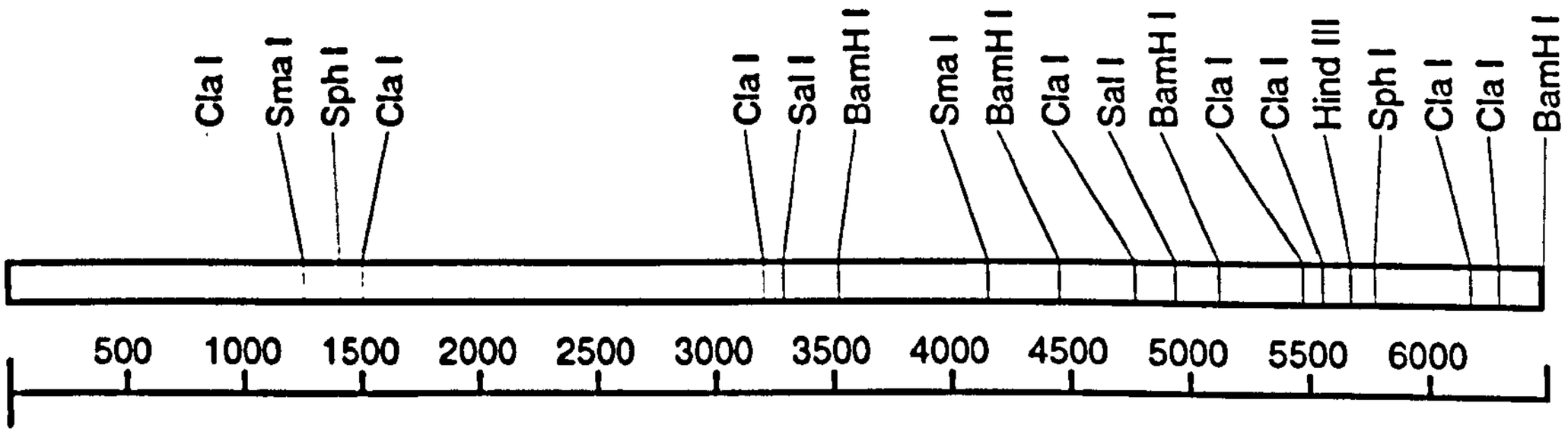


Figure 7.3 Restriction map of entire pMAT3 clone.



7.3 Discussion

Unfortunately none of the predicted proteins encoded by the open reading frames identified upstream of the chitinase gene *chiA* were similar to any proteins or putative proteins in the data bases. Furthermore, the DNA sequence was submitted to FASTA at the European Biological Institute at Cambridge via their WWW server , but this also revealed no significant match to any sequence in the data bases. It seems unlikely that this amount of DNA would be redundant; rather it is more likely that it codes for proteins of as yet unidentified function. The only conclusion that can be drawn from these data is that the chitinase gene is not a constituent of an operon and that related genes are not found immediately flanking it. The N-acetylglucosaminidase and N-acetylglucosamine permease genes must, therefore, be positioned somewhere else on the chromosome.

Section 8

Attempted cloning of N-acetylglucosamine transporter of S-symbiont (G. m. morsitans)

8.1 Introduction

As described in section 1.13.5 one approach to blocking trypanosome transmission within the tsetse fly would be to over-express a N-acetylglucosamine transporter in the S-symbiont. This is likely to cause increased uptake of free N-acetylglucosamine within the midgut of the fly, liberating the tsetse midgut lectin to defend the fly against infection with trypanosomes. Further, the enhanced ability of N-acetylglucosamine uptake in such a transformed S-symbiont is likely to give it a selective advantage over un-transformed rivals. As a first step towards overexpressing a sugar transporter in the S-symbiont, this chapter records attempts to clone the N-acetylglucosamine transporter of the S-symbiont of *G. m. morsitans*.

Sugar transporters can be of a number of different types which function quite differently one from the other and involve either a single protein or several different proteins. Their structural genes are usually organised in an operon or controlled co-ordinately. Bacterial transport systems include phosphoenolpyruvate dependant sugar phosphotransferase systems (PTS), periplasmic binding protein-dependant (BPD) ABC transporters, secondary-type systems and facilitated diffusion mechanisms.

A particular carbohydrate may be transported by different systems in different bacteria; for example, lactose is transported in *Staphylococcus aureus* by enzyme II^{Lac} of the PTS (Morse *et al.*, 1968) but by a proton symport system in the family Enterobacteriaceae (Hengge and Boos, 1983). Furthermore, the same strain (eg strains of *Klebsiella pneumoniae)* may have plasmid encoded II^{Lac} in addition to the proton symport mechanism encoded on the chromosome (Hall *et al.*, 1982). Likewise, some sugars are transported by different transport systems depending on the availability and need of the respective sugar, e.g. for most sugars such as galactose, arabinose or xylose, both BPD as well as low-affinity proton motive force dependent systems are present (Boos and Lucht, 1996). In *E. coli* N-acetylglucosamine is transported by the PTS system via enzyme II^{GlcNAc} and enzyme II^{Man} (Postma and Lengeler, 1985). Therefore, even though the S-symbiont (*G. m. morsitans*) is similar to *E. coli* it does not necessarily follow that GlcNAc in this organism will be transported by the PTS system. It is therefore necessary to review bacterial sugar transport processes in order to appreciate the complexities of

these systems and design approaches to cloning the S-symbiont N-acetylglucosamine transporter.

The PTS system is involved in both the transport of and concomitant phosphorylation of a number of carbohydrates, resulting in the intracellular accumulation of the corresponding carbohydrate phosphates. The PTS system is also involved in the movement of cells towards these carbon sources (chemotaxis) and the regulation of a number of metabolic pathways (Postma *et al.*, 1996). Each carbohydrate phosphate is the first intermediate in the catabolism of the carbohydrate, and therefore phosphorylation through the PTS provides a tight linkage between uptake and subsequent metabolism. This system consists of a number of cytoplasmic and membrane bound proteins, each of which can exist in a phosphorylated or non-phosphorylated form. As its name suggests the phosphenolpyruvate (PEP) phosphotransferase system catalyses the phosphorylation of a number of carbohydrates with PEP as the phosphoryl donor. In general, two proteins, enzyme I and heat stable protein (HPr), are required for the first step in transport, resulting in the phosphorylation of HPr at the expense of PEP (figure 8.1). Substrate specificity of the system resides in a membrane bound member(s) of the system called enzymes II, each of which can recognise a series of structurally related carbohydrates. Various forms of the PTS are found in different bacteria and in all cases enzyme I and HPr are involved indirectly in the translocation of substrates, their role being restricted to phosphoryl group donation to a variety of enzymes II. In a few organisms enzyme I and HPr are not distinct proteins but rather a single, fused protein fulfilling both roles. Conversely, enzyme II is responsible for the specificity of the system and is not always a single protein but may be comprised from one to four proteins, at least one of which is membrane bound (figure 8.1). In some cases an additional cytoplasmic protein is positioned between HPr and the membrane bound protein; this soluble phosphoryl carrier is termed enzyme IIA, in general, it interacts with only one or a few types of enzyme II. Where IIA is absent as a separate protein it is present as a cytoplasmic structural domain of the enzyme II which has an enzyme IIA-like function. In addition to this, other domains or proteins in the carbohydrate specific complex are called IIB (a second hydrophilic domain), IIC (the membrane bound domain), and IID (a second membrane bound protein found in a few PTS systems). The enzymes II span the membrane and catalyse the binding and the translocation of the respective substrate

through the membrane (Postma *et al.*, 1996). They are named using abbreviated superscripts to define the carbohydrate that they translocate, such that II^{Lac}, II^{GlcNAc} and II^{Man} refer to enzymes that transport and phosphorylate lactose, N-acetylglucosamine and mannose, respectively.

Periplasmic binding protein dependant (BPD) ATP-binding cassette (ABC) transport systems are a further category of transport system. The BPD transport systems play an important role in the uptake by the bacterial cell of a variety of substances that range from nutrients and metabolic building blocks to vitamins, enzyme co-factors and osmo-protectants These transporters are composed generally of an outer membrane diffusion pore showing substrate specificity, a soluble periplasmic substrate binding protein of high affinity and specificity, two tightly membrane bound proteins regarded as the actual permease and two peripherally, membrane-associated polypeptides associated at the cytoplasmic side of the membrane. In these systems the particular binding protein is responsible for initiating the translocation of the substrate; it also acts as a trap for the substrate, preventing it from diffusing back through the outer membrane by virtue of high affinity substrate binding. Such a high affinity system is sufficient to sustain growth of the organism for sugars that are used as the sole source of carbon. These transport systems are characterised by the presence of a highly conserved stretch of approximately 200 amino acids in one of peripherally membrane associated polypeptides, which forms a ATP-binding pocket called either the Walker motif or the Rossman fold (Boos and Lucht, 1996).

Secondary-type systems are so named because they mediate transport of solutes independently of chemical or photochemical energy; they are also known as ion coupled transporters, carriers, permeases or facilitators and require a single polypeptide that functions to catalyse substrate movement along an electrochemical gradient (Maloney and Wilson, 1996). Such a transporter can be one of three types; a uniporter, where a single substrate moves along its electrochemical gradient; an antiporter, where a substrate is taken up in exchange for efflux of anions, and a symporter, where the solute is co-transported with protons. Several hundred carrier sequences have been examined

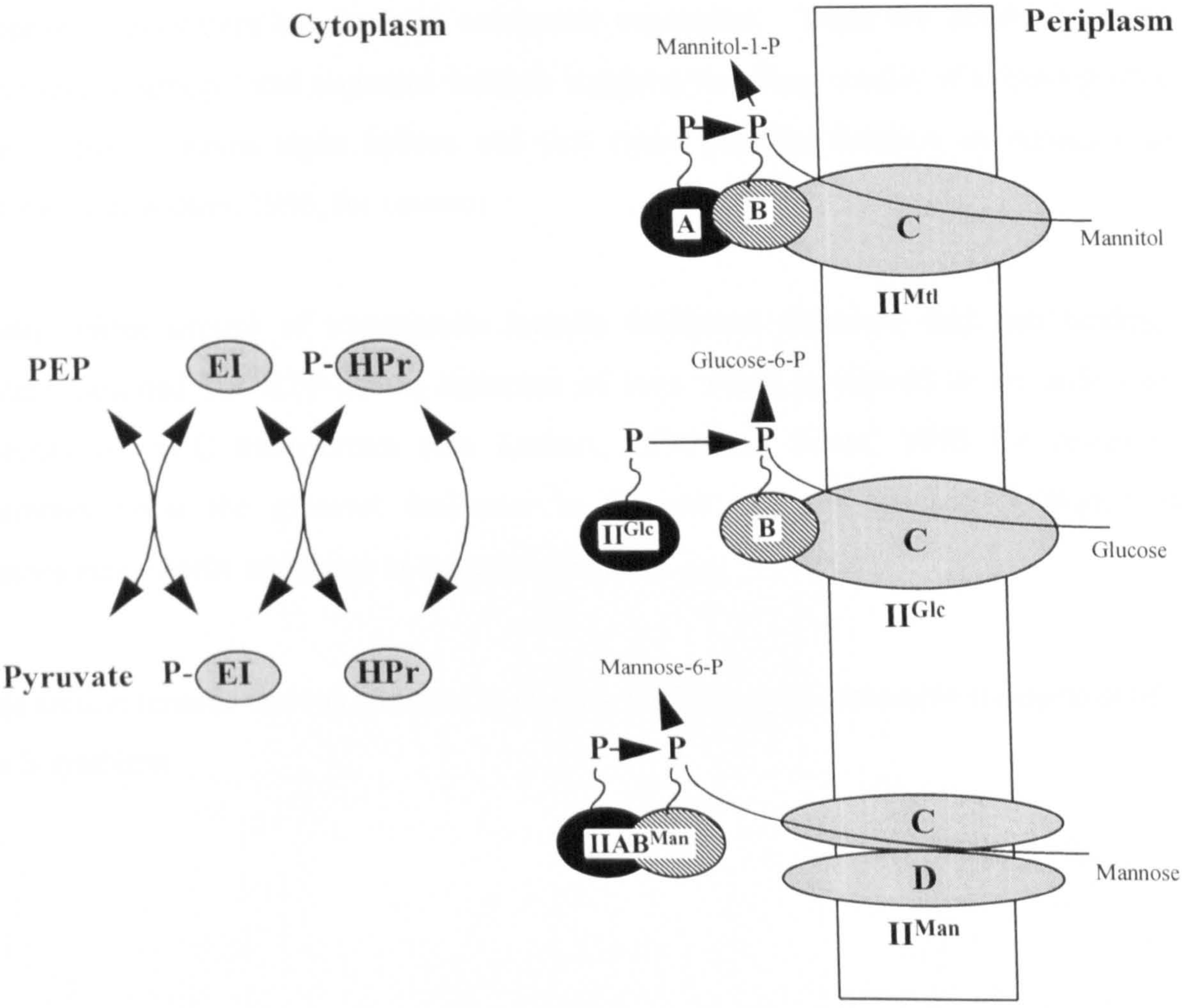


Figure 8.1 Phophotransferase systems. EI and Hpr are the general proteins for all PTSs. Of the many EIIs, only three are shown, those specific for *E. coli* mannitol (Mtl), glucose (Glc) and mannose (Man). Each contains two hydrophilic domains: IIA contains the first phosphorylation site, and IIB contains the second phosphorylation site. The membrane-bound, hydrophilic domain is IIC, but in some cases it consists of two separate proteins (IIC and IID). II^{Mtl} is specific for mannitol, II^{Glc} is specific for glucose, and II^{Man} is specific for mannose. P~ indicates the phosphorylated forms of the various proteins. (adapted from Postma *et al.*, 1996)

and together they define two groups; group I consists of 80% of all secondary type transporters and forms a super-family consisting of members from bacteria, fungi and animal cells. These transporters appear to function as monomers and have amino acid sequences that indicate the presence of 10 to 12 transmembrane alpha helices, identified by hydropathy profiles (Kyte and Doolittle, 1982). The transporters are orientated in the membrane so that the N and C termini are exposed to the cytoplasm. Group II comprises transporters largely from eukaryotic organelles. These are smaller proteins than those of group I and sequence analysis suggests that they consist of a hydrophobic core of five to seven alpha helices and that these proteins function as dimers (see Maloney and Wilson, 1996, for review).

Finally, minor groups of transporters include facilitated diffusion, and non binding-protein-mediated but ATP-driven transport of ions which is viewed as an additional category of ABC transporters (see Earhart, 1996 and Silver, 1996 for reviews). Examples being the glycerol facilitator in *E. coli* and the glucose facilitator in *Zymomonas mobilis* which act as selective channels.

This section records various attempts at cloning the N-acetylglucosamine transporter of the S-symbiont

Results 8.2

8.2.1 Effect of streptozotocin on S-symbiont

The effects of streptozotocin (stz) on cultures of *E. coli* XL-blue, *E. coli* LR2167 and S-symbiont (*G. m. morsitans*) were assessed by adding 50μg.ml^{-1} final concentration of stz, after induction of N-acetylglucosamine transport by overnight growth in nutrient broth containing 0.5% N-acetylglucosamine. Streptozotocin is a toxic analogue of N-acetylglucosamine and enters bacterial cells via transport systems for N-acetylglucosamine. Bacteria lacking GlcNAc transport systems are resistant to stz, whereas bacteria that posses GlcNAc transport systems are vulnerable. Figures 8.2 and 8.3 display the growth limiting effect of streptozotocin on *E. coli* XL-blue and the S-symbiont. However, stz has no effect against *E. coli* LR2-167 which contains a double mutation in the genes coding for the N-acetyl glucosamine and mannose transporters. This result demonstrates that the S-symbiont has a system for the uptake of streptozotocin.

8.2.2 Attempted PCR amplification of sugar transporters of S-symbiont.

A number of sugar transporter proteins from eukaryotes and prokaryotes belong to a group of transport proteins termed the secondary type transporters or the glucose transport super-family. This group of transport proteins is characterised by the members having extensive sequence homologies (Baldwin and Henderson, 1989). In particular, certain motifs occur in most of the monosaccharide transporters as well as the lactose and maltose transporters of yeasts (Henderson *et al.*, 1992). It was thought possible that the N-acetylglucosamine transporter of the S-symbiont of tsetse could be a member of this glucose transport super-family. In order to clone this sugar transporter via a PCR based strategy, a number of degenerate primers were prepared against the most homologous regions within the published sequences of the *E. coli* arabinose-H$^+$ and Xylose-H$^+$ sugar transport proteins (Maiden *et al.*, 1987). The primers were designed using the known codon bias of *E. coli* (Brown, 1991) against the motifs PESPRWL and TVDKA/FGR, which are at amino acid positions 220-227 and 334-341, respectively, of the xylose-H$^+$ sugar transporter and amino acid positions 189-196 and 317-324 of the arabinose-H$^+$ sugar transporter. Four degenerate primer sets were designed (as

described in the materials and methods section 2.14.3) to improve the chance of amplifying the desired region. The four sets of primers were then used in all possible combinations to amplify regions of DNA in *E. coli* LE392 and the S-symbiont, as described in sections 2.13.1-2. A mixture of different sized bands were amplified by this method from both the S-symbiont as well as *E. coli* LE392 using an annealing temperature of 50^0C (figure 8.3). The PCR products included amplified pieces of DNA of similar size to the expected product sizes of 405bp and 366bp for the *E. coli* arabinose-H^+ and xylose-H^+ sugar transporters, respectively. Increasing the annealing temperature of the PCR first to 52^0C and then 55^0C resulted in "fading" of all of the bands implying that no one primer set was more specific than any other.

Southern blotting and subsequent probing of the PCR products from *E. coli* LE392, with a suitable fragment of the xylose-H^+ transporter gene from *E. coli* contained in the plasmid pXylHD, resulted in hybridisation to control *E. coli* LE392 genomic DNA digest but not to any of the PCR products (data not shown). This result indicated that the *E. coli* xylose transporter gene was not amplified and therefore the amplified products from *E. coli* LE392 and the S-symbiont were unlikely to be of sugar transporters.

8.2.3 Probing of S-symbiont genomic DNA with the *E. coli* N-acetylglucosamine sugar transport gene.

S-symbiont genomic DNA was screened by hybridisation for the presence of sequences similar to the *E. coli* enzyme II^{GlcNAc} transport gene. Even at low stringency (50°C) there was no hybridisation between the radiolabelled *E. coli* N-acetylglucosamine transport gene and S-symbiont DNA whereas there was between the probe and *E. coli* LE392 DNA (data not shown). This result suggests that if N-acetylglucosamine is transported by a PTS system, then enzyme II^{GlcNAc} has little or no sequence homology with its *E. coli* counterpart. However, it is more likely that the S-symbiont GlcNAc transporter belongs to a different system.

8.2.4 Screening of gene banks for N-acetyl glucosamine transporter.

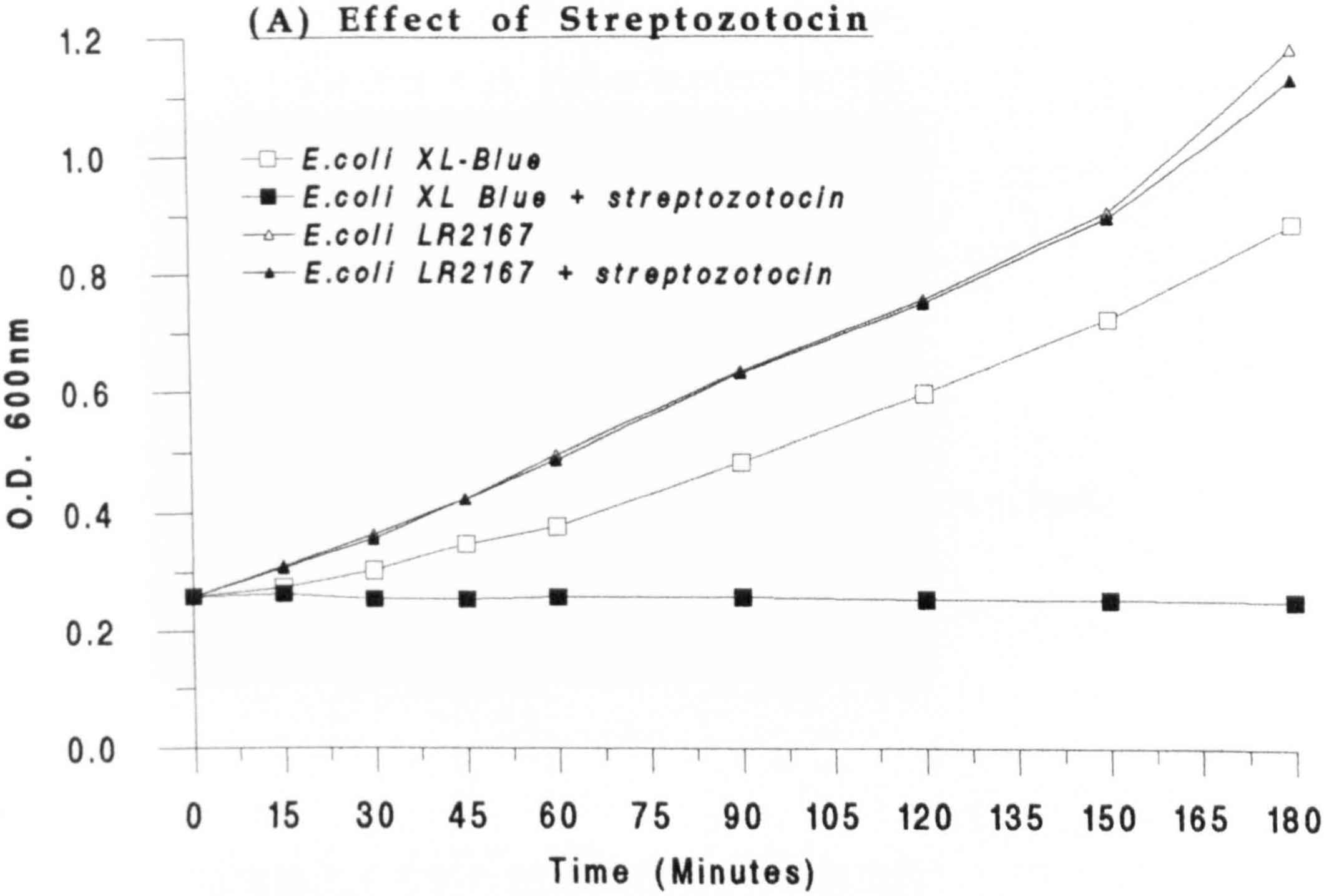
Since there was little or no homology of the S-symbiont with *E. coli* enzyme II^{GlcNAc} a mutant complementation approach was adopted. The gene banks prepared in section 5.2.1 were screened for the N-acetylglucosamine transporter of the S-symbiont by complementation of the *E. coli* mutant LR2 167. The basis of this experiment was that the *E. coli* mutant LR2 167 cannot grow on minimal agar containing N-acetylglucosamine as sole source of carbon and energy due to mutations in the genes coding for Enzyme II ^{GlcNAc} and Enzyme II ^{Man} of its PTS system. However, transformation of this mutant with the S-symbiont N-acetylglucosamine transport gene would be expected to confer on LR2 167 the ability to transport N-acetylglucosamine and so enable it to grow on minimal agar containing N-acetylglucosamine. The gene banks were screened, as described in section 2.28, on this medium. Controls included LR2 167 transformed with pUC18 as a negative control and LR2 167 transformed with pKP1.1 (which codes the Enzyme II ^{GlcNAc} of the PTS system of *E. coli*) as a positive control. These experiments yielded no LR2 167 derivatives transformed with S-symbiont genes able to grow on minimal media with N-acetyglucosamine as sole carbon source. Several thousand colonies transformed with pKP1.1 grew on the selective agar, establishing the feasibility of the approach.

Figure 8.2

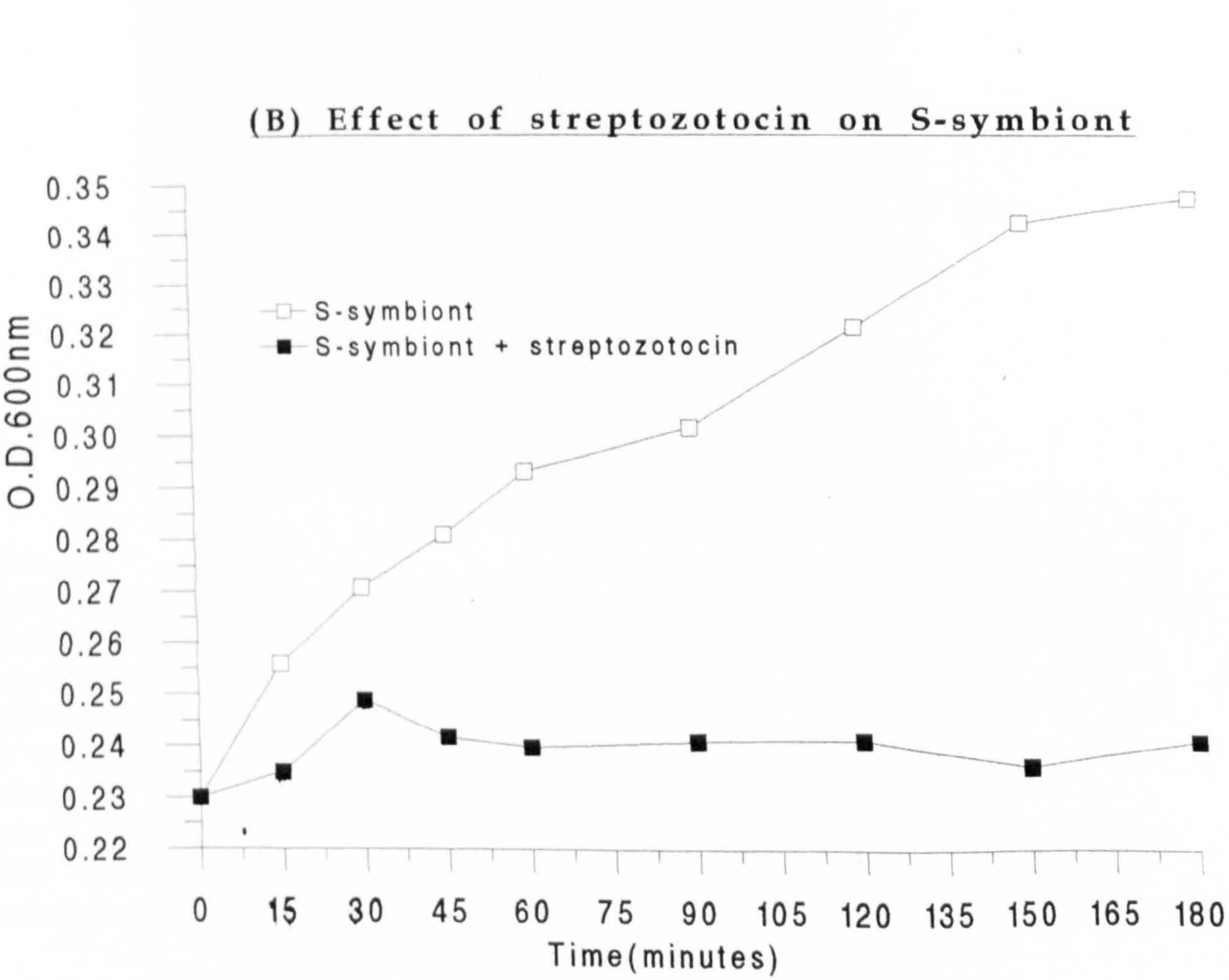
(A) The effect of the antibiotic streptozotocin (a toxic analogue of N-acetylglucosamine) on the *E. coli* strain XL Blue and the mutant *E. coli* LR2167. Streptozotocin causes a rapid growth limiting effect on XL-Blue, whereas it has no effect on LR2167. LR2167 is deficient in N-acetyl glucosamine transport due to mutations in Enzyme II ^{GlcNAc} and enzyme II ^{Man} of its PTS system.

(B) The effect of streptozotocin on the S-symbiont isolated from *Glossina m. morsitans*. Addition of streptozotocin causes a rapid growth limiting effect indicating that the S-symbiont transports N-acetylglucosamine.

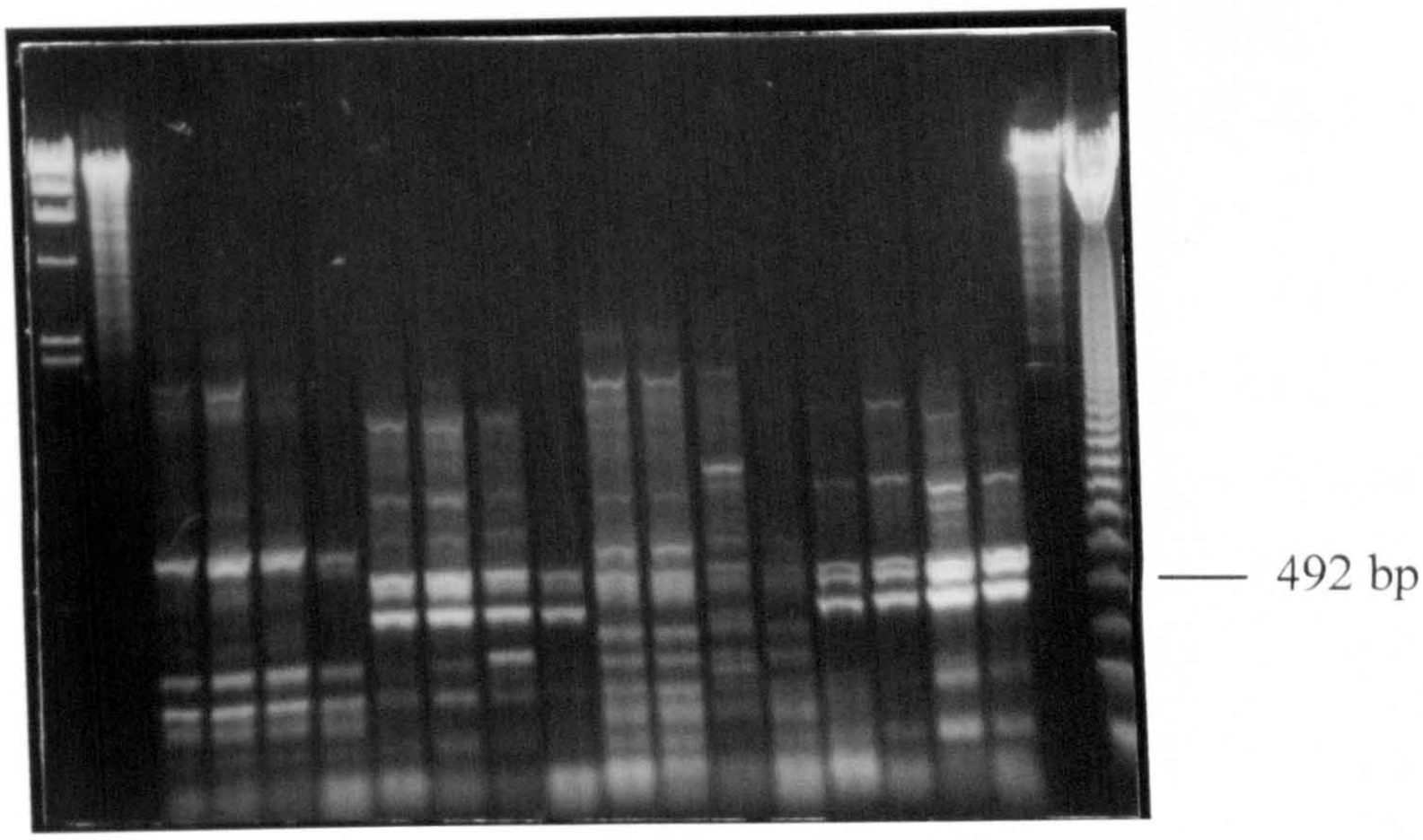
(A) Effect of Streptozotocin



(B) Effect of streptozotocin on S-symbiont



(A)



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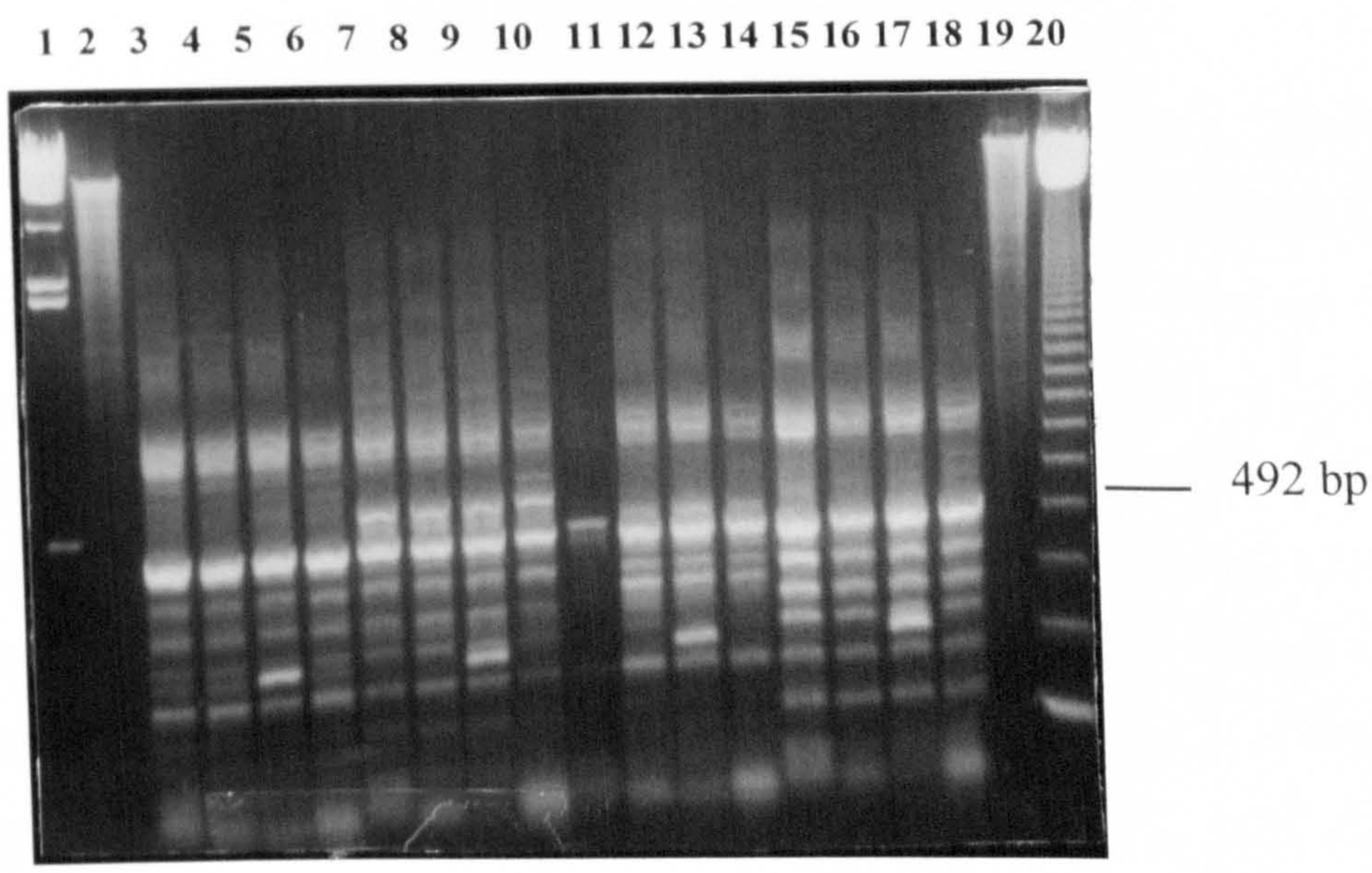


Figure 8.3

Amplification products generated using PCR primers designed from the homologous regions PESPRWL and TVDKA/FGR in *E. coli* arabinose-H^+ and xylose-H^+ sugar transporters.

(A) Amplification products from *E. coli* LE392

(B) Amplification products from S-symbiont isolated from *Glossina morsitans morsitans.*

Lanes 1, λ *Hin*dIII molecular size marker. Lanes 2 and 19, *E. coli* DNA restricted *Eco*RI. Lanes 3-18, PCR products using combinations of 4 forward and 4 reverse primers. Lanes 20, 123 bp molecular size marker.

8.3 Discussion

Two lines of evidence point to the fact that the S-symbiont possesses a functional GlcNAc transport system. Firstly the S-symbiont was shown to ferment GlcNAc producing acid (section 1), secondly, the antibiotic streptozotocin rapidly inhibited growth of the S-symbiont. Streptozotocin (2-deoxy-2-(3-methyl-3-nitrosoureido)-d-glucopyranose) is an analog of GlcNAc that is produced by *Streptomyces achromogenes* subsp. *Streptozoticus*. and enters cells via uptake systems for N-acetylglucosamine. In *E. coli* streptozotocin enters the bacterium via enzyme II^{GlcNAc} and is concomitantly phosphorylated (Ammer *et al.*, 1979). Streptozotocin 6-phosphate cannot be metabolised and generates highly toxic and mutagenic diazomethane that kills cells. (Postma and Lengeler, 1985). The fact that streptozotocin inhibits growth of the S-symbiont demonstrates that this bacterium has an uptake system for GlcNAc. Therefore approaches were developed to clone this gene with a view to overexpressing it in the S-symbiont

The attempted PCR amplification of transporters belonging to the secondary type systems was unsuccesful. A number of amplification products were recovered following amplification using the degenerate primers designed against conserved sequences of the xylose-H^+ and arabinose-H^+ sugar transporters. DNA fragments amplified using S-symbiont and *E. coli* template DNA were screened with a radio-labelled fragment of the *E. coli* xylose-H^+ gene. No hybridisation was observed between the labelled probe and any of the amplified DNA fragments indicating that none of the amplified DNA fragments were of the xylose-H^+ gene. This experiment served as a positive control, since the degenerate primers were designed to target regions of the *E. coli* xyloseH^+ gene that are homologous with the genes of a large number of other secondary type transporters. Since not even the xylose H^+ gene of *E. coli* was amplified using the degenerate primers, it was unlikely that any of the amplified products from either bacteria were of secondary transport type genes; what was amplified is unknown. In addition to this, even if one or more of the PCR products did constitute fragments of a transport gene, the entire gene would then have to be cloned, using the fragment as a probe, from a library of genomic DNA. It would then be necessary to determine the specific function

of the cloned gene via amino acid homology to other transport genes and then complementation analysis.

Since the S-symbiont is related to *E. coli* (Section 1) where N-acetylglucosamine is transported by the PTS system via enzymeII^{GlcNAc} and enzymeII^{Man}, (Postma and Lengeler 1985) it is possible that N-acetylglucosamine is also transported by a PTS system in the S-symbiont. S-symbiont genomic DNA was screened by hybridisation with the labelled *E. coli* enzymeII^{GlcNAc} gene for similar sequences. There was no hybridisation detected between the labelled *E. coli* DNA and the S-symbiont DNA even when screened at low stringency. This suggests that even if N-acetylglucosamine is transported by a PTS system in the S-symbiont, the S-symbiont transport gene shares little or no homology with its *E. coli* counterpart.

Finally a mutant complementation approach was adopted to clone the S-symbiont GlcNAc transport gene. A mutant *E. coli* strain LR2-167 (unable to transport N-acetylglucosamine due to mutations in the genes II^{GlcNAc} and II^{Man}) was used to screen the available S-symbiont genomic libraries for the N-acetylglucosamine transport gene by complementation of the transport mutations. Unfortunately this approach was also unsuccessful; even though all six gene banks were screened, no colonies were obtained on minimal agar containining N-acetylglucosamine as the sole carbon source, whereas LR2-167 transformed with the plasmid pKP1.1 containing the enzymeII^{GlcNAc} gene of *E. coli* did grow on identical medium. There are a number of possible reasons why this approach failed to isolate the S-symbiont GlcNAc transport gene. If the S-symbiont GlcNAc transport gene is part of its PTS system, then the complementation experiment relied on the ability of the gene from the S-symbiont being expressed in *E. coli*. Additionally the approach also relies on the assumption that the components of the PTS systems in both bacteria are interchangeable. If either assumption is wrong the experiment will necessarily fail. However, the enteric HPrs are very similar and those from *E. coli* and *Salmonella typhimurium* are identical (Byrne *et al.*, 1988). Additionally, the deduced amino acid sequences of the enzyme I from *E. coli* and *Salmonella typhimurium* differ in only 16 residues and are functionally interchangeable (Postma *et. al.*, 1996) therefore there was every reason to hope that this approach would work. If the S-symbiont enzymeII^{GlcNAc} gene does not exist N-acetyglucosamine must be

transported by a different type of transporter. The complementation approach should still work, but it may be difficult to recover the full system in one clone if a number of genes are required for the particular transport system to function. Another possible reason for the failure of this approach is that the particular gene may not be represented in the gene banks.

Future attempts to clone this gene could involve the production and subsequent complementation of GlcNAc transport mutants of the S-symbiont itself rather than a near relative. This would ensure the functionality of the system; alternatively, transposon tagging could be utilized to locate the gene(s) in the S-symbiont chromosome, prior to cloning.

Section nine

General discussion

General discussion

9.1 Summary

This project started with the aim of looking in detail at the chitinolytic system of the tsetse S-symbiont. A great practical aid to the project was the development of a suitable *in vitro* culture system consisting of growth on blood-agar under reduced oxygen conditions. This made it both easier and less expensive to grow large numbers of S-symbiont from which to isolate genomic DNA for gene library construction. This study also has given direct evidence by enzymology that the S-symbionts isolated from *Glossina m. morsitans*, *Glossina p. palpalis* and *Glossina austeni* possess chitinase systems consisting of exochitinase, endochitinase and N-acetylglucosaminidase activities. This system appears to be very similar between the different strains of S-symbiont isolated from different species of *Glossina.* However, the S-symbiont isolated from *Glossina m. morsitans* displayed higher N-acetylglucosaminidase, exochitinase and endochitinase activities than the S-symbionts isolated from the other two species of fly. Additionally, the gene responsible for the major exochitinase and endochitinase activities of the S-symbiont isolated from *Glossina m. morsitans* has been cloned and sequenced. Sequence analysis predicts that the gene encodes a protein of 76 kDa that displays highest homology to *Aeromonas caviae* ChiA chitinase and includes the conserved active site residues of family 18 of the glycosylhydrolases.

9.2 Investigating the S-symbiont

The microaerophilic nature of the S-symbiont is reflected in the niche that it occupies within the tsetse fly. It is found dividing within tsetse cells where it would be expected to be in the presence of a rich source of catalase and superoxide dismutase which destroy toxic oxygen radicals. Biochemical tests highlighted the absence of catalase which is normally found in bacteria belonging to the Enterobacteriaceae. It is therefore hypothesised that this particular enzyme is redundant in the particular niche that the S-symbiont occupies and has been lost through the course of evolution. Loss of genes through evolution is fundamentally easier to envisage than their acquisition. The S-symbiont grew well

on blood agar in a reduced oxygen environment but grew poorly on nutrient agar under the same conditions and also grew poorly on blood agar at normal oxygen conditions. The growth of the bacterium in culture could possibly be improved further by altering its genetic makeup by including genes for the production of catalase and/or superoxide dismutase However, additional nutrients may also be supplied by the blood agar that enhance its growth. The ability to culture the bacterium under normal atmospheric oxygen conditions would enhance its suitability for study and manipulation.

Of paramount importance in the short term, with respect to production of manipulated S-symbiont and in the long term with the aim of producing pseudo-transgenic tsetse, is the ease with which the S-symbiont can be transformed. Not only is a reliable transformation system needed to introduce foreign genes in order to assess their expression and activities within the S-symbiont, but also for the production of specific mutants by allele exchange. This project highlighted the difficulty encountered when attempting to transform this bacterium: the electrotransformation protocol used failed to achieve transformation and divalent cation treatment and heat shock achieved only a very low transformation efficiency on a single occasion. All attempts made to produce mutants lacking chitinase were unsuccessful, almost certainly because of the low transformation efficiency. The bacterium was transformed, albeit at low efficiency, with a ColE1 based vector and has previously been transformed with a RSF1010 based vector which demonstrates that this approach is at least feasible (Beard *et al.*, 1993). The current difficulties encountered in attempts to transform the S-symbiont may be due to the possession of a restriction modification system by the bacterium (C.Dale, Division of Molecular Genetics, Glasgow University, personal communication). If this is the case, mutants could be selected that are deficient in production of restriction enzymes which should be considerably easier to transform. The isolation of a phage from the S-symbiont (C.Dale, personal communication) may be the key to the routine transformation of this bacterium, as it may be possible to manipulate it to serve as a transformation vector. Study of this phage is presently being pursued, with this in mind, at Glasgow.

9.3 Steps towards pseudotransgenic tsetse

The cloning of the chitinase gene of the S-symbiont and its overexpression in *E. coli* presents a landmark in the study of this particular organism. It is not only the first functional gene that has been cloned from this bacterium, but its overexpression in *E. coli* demonstrates the feasibility of using *E. coli* for the study of S-symbiont genes and the proteins that they encode. The gene is of particular importance because of the implication of the S-symbiont chitinase system in rendering the tsetse fly susceptible to infection with pathogenic trypanosomes.

9.4 Model of S-symbiont promoted susceptibility to infection.

A model has been put forward to describe the way that the S-symbiont generates vulnerability in the tsetse fly to infection with pathogenic trypanosomes (Figure 1.18). The model is based on biochemical events during the pupal period of fly development. The tsetse fly is viviparous and acquires S-symbiont infection maternally probably through the milk gland secretions in the uterus (Bonnanfant-jais, 1974), S-symbionts consequently invade the midgut of the developing larvae (B in figure 18.1). During the pupal stage S-symbionts multiply within the larvae (Welburn, 1991; Maudlin personal communication). The S-symbionts are believed to be actively secreting chitinase during this time in order to produce N-acetylglucosamine and glucosamine from the chitin lining of the midgut wall of the fly as a source of carbon and energy. The free sugar concentration increases and blocks a midgut lectin that has specificity for these sugars (Ibrahim *et al.*,1984; Maudlin and Welburn, 1987; Welburn 1991) that normally functions in a defensive mode against pathogenic trypanosomes. As the fly takes its first bloodmeal it is unaware that its defences are down, if the first animal that it feeds on is a reservoir of trypanosomes the fly becomes infected. Thus the S-symbiont chitinase promotes tsetse fly infection with trypanosomes. However flies which do not acquire S-symbionts from their mothers (A in figure 1.18) do not have chitinase secreted in their midgut and hence the defensive lectin is free to destroy invading trypanosomes if it first bloodmeal is infected. After the first bloodmeal, bloodmeal serum activates the secretion of midgut lectin (Gingrich *et al.*,1982; Stiles *et al.*, 1990) to such a level that it is unlikely that the fly ever will become infected. whether it carries S-symbionts or not.

9.5 Investigating chitinase involvement in generating tsetse susceptibility.

Although the involvement of the S-symbiont chitinase gene in this process of producing susceptibility to trypanosome infection in tsetse flies was not addressed by this thesis, the work reported here provides the groundwork from which to address this question, as well as a point from which to develop pseudo-transgenic tsetse. The cloning of the chitinase is of central importance to the production of chitinase minus mutants by allelic exchange and a preliminary construct has been made for this purpose. Once the problem of the low transformation frequencies obtained with this bacterium have been overcome, S-symbiont mutants lacking chitinase will be produced. These mutants could be introduced into apo-symbiotic tsetse and maintained by antibiotic administered via the tsetse bloodmeal. The susceptibility of these tsetse flies to trypanosome challenge could then be ascertained and compared with that of S-symbionts overexpressing the chitinase, as well as the controls comprising the wild type symbiont.

Antibodies could be generated against purified S-symbiont recombinant chitinase. These antibodies could then be used to detect amounts of chitinase present during the pupal period of flies infected with S-symbionts to see whether there is a build up of this enzyme in the midgut during pupal development. Similarly oligonucleotide primers designed against the chitinase gene sequence could assess expression of the chitinase during this period by reverse transcriptase PCR.

Analysis of chitinase production by the S-symbiont isolated from three different species of tsetse demonstrated that all the strains studied produced a similar profile of constitutive chitinase activity, as determined by SDS-PAGE, subsequent renaturation and activity staining. Interestingly, the S-symbiont isolated from *G.m. morsitans* produced greater endochitinase activity than the S-symbionts from the other two strains studied (*G. austeni and G. palpalis)*. The ability to transmit pathogenic trypanosomes varies between different tsetse species. Members of the *palpalis* group are generally most refractory whilst species in the *morsitans* and *fusca* groups exhibit varying levels of susceptibility, which may reflect, in part, the greater chitinase activity of the *G. m. morsitans* S-symbiont.

9.6 Prospects for manipulation. Turning the tables

In addition to colonising the mid-gut of the fly, the trypanosome must make its way to the salivary glands or mouthparts of the fly and mature into mammalian infective forms before the infection can be passed on to the next host. This maturation is dependant on a signal from the midgut lectin and if this lectin is blocked by continually feeding glucosamine and N-acetylglucosamine with the fly's blood meal. Thus there are two points in the cycle of trypanosome infection that are candidates to blocking trypanosome transmission. Pseudotransgenic tsetse overexpression of an N-acetylglucosamine sugar transporter may remove free sugars in the midgut during the pupal period of the fly and hence not inhibit the tsetse defensive lectin. The second point is to block maturation off midgut trypanosome infections by high expression of the S-symbiont chitinase. This would produce free sugars blocking the lectin signal for trypanosomes to mature hence the tables would be turned. Instead of the S-symbiont promoting transmission of pathogenic trypanosomes it would block transmission.

Sequence analysis of the chitinase gene indicates the position of various domains within the chitinase by comparison with other chitinases. This comparative analysis suggests that S-symbiont ChiA consists of a catalytic domain, a domain which shows homology to other chitin binding domains found in various chitinases and a domain that may be involved in its ability to form dimers. It would be interesting to analyse the effect that the various suggested domains in the actual chitinase have, on the susceptibility to infection in tsetse. S-symbionts could be introduced into tsetse with manipulated chitinases. Such manipulations could involve deletion of the chitin binding domain or the domain hypothesised to be involved in dimerisation. The effect of these changes on chitinase activity could be assessed with regard to susceptibility of tsetse to trypanosome infection. Such subtle approaches to increasing chitinase activity may be more effective than just high level expression, since high level expression of chitinases within insects which possess a large amount of structural chitin could be deleterious to the individual insect.

Maternally inherited S-symbionts appear to confer a selective advantage to the tsetse host by increasing pupal survival by possibly weakening the pupa wall, leading to a larger proportion of insects surviving emergence (Baker *et al.*, 1990). It is possible that this selective advantage is dependent on S-symbiont produced chitinase breaking down the structural chitin in the pupa. A similar observation has been observed in *Tetanops myopaeformis* infected with *S. marcescens* (Iverson *et al.*, 1984) which is known to secrete chitinase. The use of chitinase mutants could address this question of whether this effect is due to the S-symbiont chitinase. Additionally, subtle overexpression of the chitinase could both block maturation of trypanosome infections as well as further increasing puparial survival.

It is unfortunate that the N-acetylglucosamine gene was not cloned during this period of study. Recovery of the GlcNAc transport gene of the S-symbiont is a high priority. Once isolated, high level expression of this transporter must be placed high on the agenda of approaches for producing pseudo-transgenic tsetse incapable of transmitting disease. Sugar transport has been proposed as the rate limiting step for sugar metabolism (Postma and Lengeler, 1985) and therefore high level expression of this gene may confer a selective advantage over non-manipulated S-symbionts. It would also be interesting to ascertain if the *E. coli* enzyme II^{GlcNAc} gene is expressed in the S-symbiont and whether high level expression would increase sugar uptake. This could be ascertained using radiolabelled GlcNAc (Peri and Waygood, 1988).

9.7 A wider picture

The infection rate of flies with trypanosomes in the wild is generally very low, rates being recorded in one study as: *Trypanosoma vivax* 6%, *Trypanosoma congolense* 2% and no *Trypanosoma brucei* infections (Maudlin, 1991 citing Pires *et al.*, 1950). The relative fly infection rates between different trypanosome species can largely be explained by the relative complexities of the life cycles within the fly; *T. vivax* has the simplest life cycle within the tsetse host by being confined to the mouthparts whereas *T. congolense* and other trypanosome species

go through a midgut stage before returning to mature in the mouthparts (Maudlin, 1991) (*T. vivax* and *T. congolense* are non-pathogenic to humans whereas the *brucei* group includes two human pathogens; *T. b. rhodesiense* and *T. b. gambiense* which are morphologically identical to *T. b. brucei* which is pathogenic to livestock). The general low percentage of infection of flies with trypanosomes in the wild has now been studied in depth and has been related to the defence mechanisms of the individual fly species which are based on trypanocidal lectins (Welburn *et al.*, 1989). *Glossina palpalis* has been shown to have a lectin defence system which is based on two lectins, one with specificity against N-acetylglucosamine and one with specificity against galactose. Clearly, overexpression of a GlcNAc transporter in this fly would not be expected to totally block the defensive capabilities of this particular fly which is reflected in its infection rate with trypanosomes being generally lower than *morsitans* and *fusca* groups. However, the lectin system in *G. m. morsitans* is only based on a single lectin with specificity to glucosamine and N-acetylglucosamine, and this species of fly is much more susceptible to infection than the *palpalis* group. The mechanism of maturation of trypanosome infections from the midgut to mature forms in the salivary glands is likewise not automatic and profoundly influenced by fly species. This has been supported by experiments looking at the transmissibility index TI, (defined as the percentage of mature trypanosome infections verses midgut trypanosome infections) of different species of flies. *Glossina m. morsitans* promotes a TI of 100% in *T. congelense* stock 1/148 (Welburn and Maudlin, 1989), whereas *G. p. palpalis* rarely produces a mature infection with the same stock (Welburn *et al.*, 1994). Maturation is also affected by trypanosome species and stock: *T. brucei* infections show great variation in TI between stocks; human infective *T. b. rhodesiense* producing significantly lower TIs than non human infective *T. b. brucei* (Maudlin and Welburn, 1994). Maturation of *T. congolense* stocks can also be great in the same species of fly (Maudlin and Welburn, 1988). Since it has been shown that continual blocking of the insect defensive lectin by feeding N-acetylglucosamine/glucosamine can block maturation of trypanosome infections from midgut to salivary gland infections, overexpression of chitinase may block trypanosome maturation. However, this will need to be determined

empirically for different types of trypanosome fly combinations since a number of variables are obviously involved.

Appendix A

Next two pages

Biophysical properties of ChiA

Molecular Weight 76371.90 Daltons
695 Amino Acids
55 Strongly Basic(+) Amino Acids (K,R)
87 Strongly Acidic(-) Amino Acids (D,E)
222 Hydrophobic Amino Acids (A,I,L,F,W,V)
211 Polar Amino Acids (N,C,Q,S,T,Y)

4.658 Isolectric Point
-30.149 Charge at PH 7.0

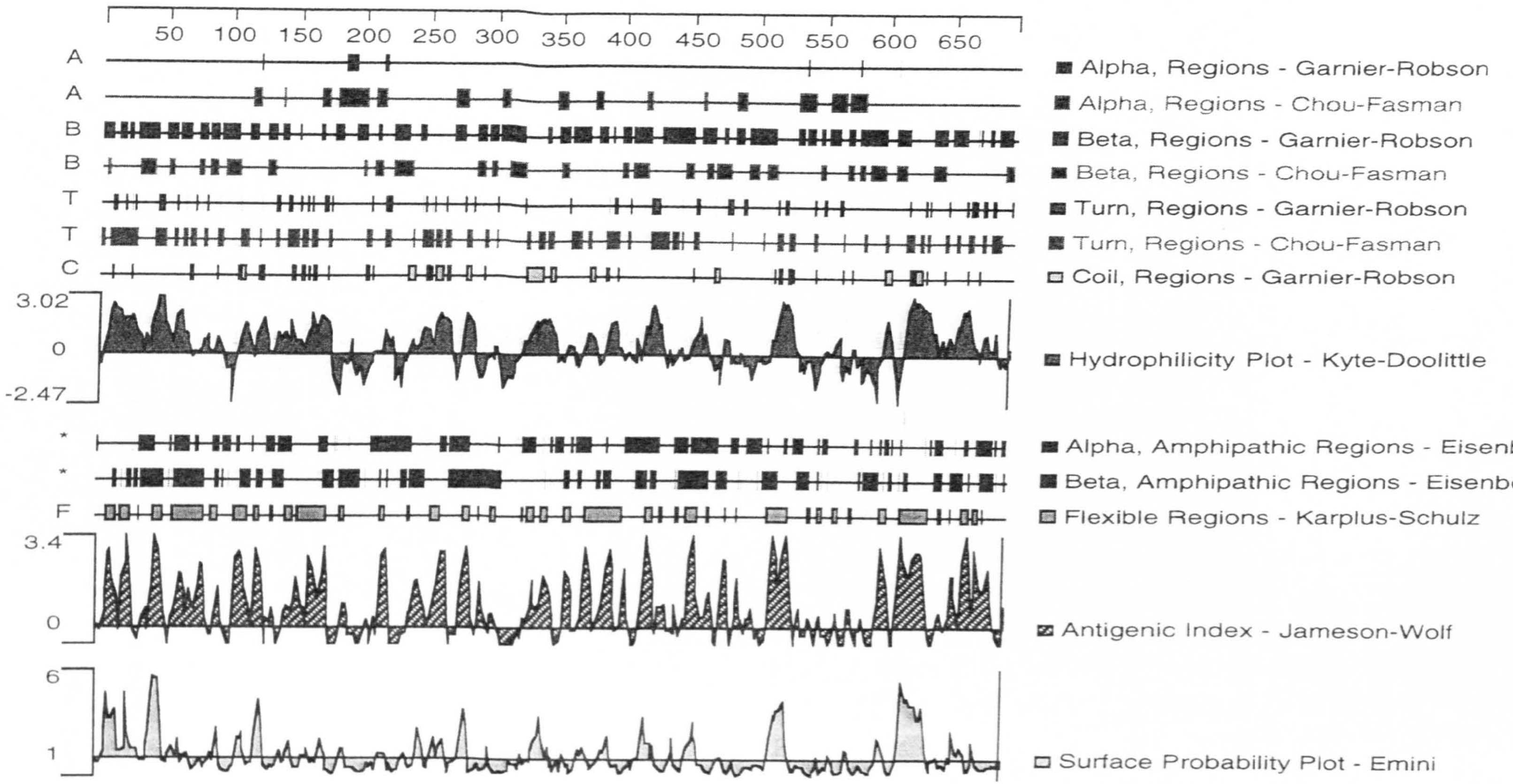
Total number of bases translated is 2088

% A = 25.34	[529]	
% G = 26.20	[547]	
% T = 24.04	[502]	
% C = 24.43	[510]	
% Ambiguous = 0.00	[0]	
% A+T = 49.38	[1031]	
% C+G = 50.62	[1057]	

Davis,Botstein,Roth Melting Temp C. 85.42
Wallace Temp C 7310.00

Codon usage:

gca	Ala(A)	7	# cag	Gln(Q)	21	# uug	Leu(L)	6	# uaa	Ter(.)
gcc	Ala(A)	25	# ---	**Gln(Q)**	30	# ---	**Leu(L)**	42	# uag	Ter(.)
gcg	Ala(A)	17	# gaa	Glu(E)	29	# aaa	Lys(K)	25	# uga	Ter(.)
gcu	Ala(A)	9	# gag	Glu(E)	8	# aag	Lys(K)	11	# ---	**Ter(.)**
---	**Ala(A)**	58	# ---	**Glu(E)**	37	# ---	*Lys(K)*	*36*	# aca	Thr(T)
aga	Arg(R)	1	# gga	Gly(G)	4	# aug	Met(M)	8	# acc	Thr(T)
agg	Arg(R)	1	# ggc	Gly(G)	22	# ---	**Met(M)**	8	# acg	Thr(T)
cga	Arg(R)	1	# ggg	Gly(G)	13	# uuc	Phe(F)	21	# acu	Thr(T)
cgc	Arg(R)	5	# ggu	Gly(G)	23	# uuu	Phe(F)	9	# ---	**Thr(T)**
cgg	Arg(R)	5	# ---	**Gly(G)**	62	# ---	*Phe(F)*	*30*	# ugg	Trp(W)
cgu	Arg(R)	6	# cac	His(H)	3	# cca	Pro(P)	8	# ---	**Trp(W)**
---	**Arg(R)**	19	# cau	His(H)	10	# ccc	Pro(P)	7	# uac	Tyr(Y)
aac	Asn(N)	13	# ---	**His(H)**	13	# ccg	Pro(P)	16	# uau	Tyr(Y)
aau	Asn(N)	20	# aua	Ile(I)	3	# ccu	Pro(P)	6	# ---	**Tyr(Y)**
---	Asn(N)	33	# auc	Ile(I)	16	# ---	**Pro(P)**	37	# gua	Val(V)
gac	Asp(D)	18	# auu	Ile(I)	15	# agc	Ser(S)	17	# guc	Val(V)
gau	Asp(D)	32	# ---	**Ile(I)**	34	# agu	Ser(S)	11	# gug	Val(V)
---	**Asp(D)**	50	# cua	Leu(L)	3	# uca	Ser(S)	5	# guu	Val(V)
ugc	Cys(C)	3	# cuc	Leu(L)	3	# ucc	Ser(S)	12	# ---	**Val(V)**
ugu	Cys(C)	6	# cug	Leu(L)	18	# ucg	Ser(S)	8	# nnn	???(X)
---	**Cys(C)**	9	# cuu	Leu(L)	4	# ucu	Ser(S)	4	# TOTAL	
caa	Gln(Q)	9	# uua	Leu(L)	8	# ---	**Ser(S)**	57	#	


50 100 150 200 250 300 350 400 450 500 550 600 650
A
A
B
B
T
T
C
Alpha, Regions - Garnier-Robson
Alpha, Regions - Chou-Fasman
Beta, Regions - Garnier-Robson
Beta, Regions - Chou-Fasman
Turn, Regions - Garnier-Robson
Turn, Regions - Chou-Fasman
Coil, Regions - Garnier-Robson
3.02
0
-2.47
Hydrophilicity Plot - Kyte-Doolittle
*
*
F
Alpha, Amphipathic Regions - Eisenberg
Beta, Amphipathic Regions - Eisenberg
Flexible Regions - Karplus-Schulz
3.4
0
Antigenic Index - Jameson-Wolf
6
1
Surface Probability Plot - Emini

Appendix B

Next page

N-terminal analysis of S-symbiont ChiA

Query Information

ORIGIN Gram-negative bacterium
BEGIN
>MYSEQ
MSSQLIQKDQ YSDESYQYDG FDPKTNDSAY SYTSARVMKR VYNKYDTKNK
PKVFGYYTDW GQYDGRALSS PPSGSVDVGS RGRGVDFSQL SPTAYDKIIF
GFTGIVGDKG ANQYKIEQAA AWTGKKQYEM TILDPWGDCQ AYFNNGFSSY
KDFGFGPGTT YNGGSQEDCF KESHPNLQGV LGALLALKKK AALAGHDLAL
SFSVGGWTMS EIFHEMVKSD QAINTFVSSI VDFFQRFPSF SEIDIDWEYP
NAAGAGNPHG PEDGANYQKL IAALRQAFNS HNRQDIKISI ASSANVDVLQ
HSNIKGLLAA GLYGINVMTY DFFGTPWHEG LTNHTNLYKT EHSSYSLEEA
VTYLLEQGVD PDVINVGYAG YSRSAKGAEI SSFSPLKGTY EGNDTTVGTF
ESGCVIEWYDV LYNYLDLENK SGRNGYQVYT DDVACADYLY SPTAKVFHSI
DTPRSVREKA RYVIEKGLGG IFTWTIDYDN GLLVNAAREG LGCPIVDKVI
DMSPFYFKGI NITGEDEGKP DEPNTPDKPA AAPVAKVEIK AFAGSSLLFC
GQQSVNAACY EWSATQGAVI AAPHAEQTAV VLPNVSVDTL ISTTLAVINE
SGERATAVFA LTVVPKDDTD EQPETPDEPE TPSQYQQWIA TQIYTEGNLV
SHKGVDYRAN HWSQGDEPVC LPPPDSMVSR GRCWLPDPIA LYRFG

Result Information

PSORT --- Prediction of Protein Localization Sites
version 6.4(WWW)
MYSEQ 695 Residues
Species classification: 2

*** Reasoning Step: 1

Lipop: Examining lipoprotein consensus (Klein et al.:modified)
Possible modific. site: -1 CRend: 8
McG: Examining signal sequence (McGeoch)
Length of UR: 3
Peak Value of UR: -1.44
Net Charge of CR: 0
Discriminant Score: -27.34
GvH: Examining signal sequence (von Heijne)
Signal Score (-7.5): -7.92
Possible cleavage site: 35
>>> Seems to have no N-terminal signal seq.
Amino Acid Composition of Predicted Mature Form:
calculated from 1
ALOM: Finding transmembrane regions (Klein et al.)
count: 1 value: -0.27 threshold: 0.0
INTEGRAL Likelihood = -0.27 Transmembrane 579 - 595 (579 - 597)
PERIPHERAL Likelihood = 3.29
modified ALOM score: 0.55
Rule: cytoplasmic membrane protein

*** Reasoning Step: 2

----- Final Results -----

bacterial inner membrane --- Certainty= 0.111(Affirmative) < succ>
bacterial periplasmic space --- Certainty= 0.000(Not Clear) < succ>

Appendix C

S-symbiont upstream sequence

BASE COUNT 885 A 834 C 895 G 898 T 8 OTHER

ORIGIN -

1 GCGCGTTTTA GCGCTGACAT GAATGTGGCG GAAATCATTT TACCGCCGGT AACGCCTGTA
61 AAAACATTGT CCAGCGCCAA ACGCAAAACA TTTTCGCCGC CGTCAAATTT GGTTAATGCG
121 GCCAGCACGC GTTCCACGCC ATGATCCTGG ATTAATGCCG GCCCGATATA GTGTGTAATA
181 CCGGCGGCGA TTTTCCGCGC CAGTTCACCC TGCCCTTTAG ATTGTAAAAT GAGATCAACC
241 AATGGCGCCA ACCCGTTTTC GGCCTGCGCT CGGGTATTAT CGAACAGCGC TGATCTGCCC
301 TGATTAACAA TCTCTGCGCT ACGTTTTCCT AAACTCTTAT TGCTTATGAC ATAATGCTGC
361 GCCAGCGTGA TCGTATCGGA AATGATATTT TTAGCCAGCT TGTCTCGTGT AATTGAACCC
421 AGGCTGATGC GTTTGGTTAA GGCATTTAAT TTGCTATTGC TGTCCATGCA GCGATAAATG
481 AGGACACGAA GGCCAAATTT GGCGATGGTG GCATTATTCA TGGCGTCAAG GTCCTTTTAA
541 AGCGTTGGCC GGCGGAAGTA TCCAATAATG ACGCCTGGGT TGCCTGACCT ATTTGGGGTA
601 AATTCTCTTT CCAGTATTGC GCTTGATCAA TTAGCGTTTC GACGCACTTC AGGGCGGCTT
661 CTGCCGATAA CAGGGCTAAA TCCATCAAGC AAAAGAGGAT GATTCGCTGG CCGCCGGCGT
721 TGTANCCAAA CCAGCAGCGA TGTGTGGCGA TACCGAATAA ATGGATCTGC AAAAGAGAGG
781 CAAAAAAATC ATCCGACAGC GGTTGGGTAA TTTGCATCAC CGGCGAAAAG AGATACACGT
841 GGCTGTCATG ATTTTCAAAC GTAATATCGA TATTATCGTC GAAACGAATA GTGCAGCCAC
901 CGCTTTGCAG TGCGTCGTTA ATATTTAAAT GCGCATCCGC ATTGAGTGAT TGTAGGAGTT
961 CAGAAAAAGT CATCGCGTTA TCTCTGGTCA GTTCGCACAT TTTACCGCCC GATGGTCGGC
1021 AAATATGCTC AAGAACAAGT GTTTATTTAA AATAATATAA TTAATTAAAT AATTATATAA
1081 AATTTCGATT ATATTTTTTT CAAGCACAGC GATGCGCAGC CAGACAGGGC CCCTTGCAGC
1141 TGACAGCCAA CATAGTCAGG CATTGGGAAT TTGACATGAC TGTGCGAGAT TACCGCTCAT
1201 CCTGCGTTGC CCTATTCTGA GCGAGTGAGA TGAAACGGGC GTCGAGGGAA GGAGGGATTT
1261 GGCTTGGTCA ATTACGCGCC CGGGCCCGGC GCAGGACAGA GAATGAGGAA ATAAAGTGCA
1321 GCGCCCGATC GGAGTAAGCC GCCATTATAG GTTGTTGGAG AACAGCTCCG CCATTTTCAC
1381 TGCATCATAA TAACCCCAGA TTTCGCTGAT TTTATCGTTG CGCATGCGTA TTACCTAGCA
1441 GTAGTGCATG TTGAAGTCTA CGCCATTACT CCCTCTTCTG CATGAAATTG CAGGCAAGCC
1501 ATGTTCCCCT CGACGATGAT CGATTCGACG ACGGGACGGG GCCAGGAAGC CAGCCTGTAG
1561 TCGAGCTTGT TTATCACCTG CTCGCGATAG GCATGTATAC CGTGTGATAT GTTCCGGCTA
1621 CGGGTGACGT TCCCATGGAG TTAAACACTA CATCGTCATG CAGCGCGGCA AAAAAACCGC
1681 CGTCCAGCCG CGCGGGCGGC GATGGAGGAA AAAAGAGATT CAAGAAACCT GCGGTAATCA
1741 GTATGGGATG AATGCATTTT ACTCTCTCCT TGCTCGGCGC CGGTTCAGTC GCGTGAGTGC
1801 AGTACTTCGT TCAAGCCGTC GGTCAAAAGT CGGAGCACAC ACGACCCGCC ATCTACCGGC
1861 AATAGCGTGC AGCGTTTTAT TTTCCATTGC TCCTGATGAC GCTCTAGCTC CCAACGATTG
1921 ACAACGGCAC GGTGGTATTC TGTACCCGGT TGAAGTGCTG TGATTAGCCA GCGTGCGGCG
1981 TTTACCTTCC TGATCAAGCT ATATAATCAG AAGATAGGAA ATCACCACCG CGCTATCACC
2041 GTTTAGAAAG ATAAGGGGCA ACCCGGTAAA ATGTGCCAAC CCGCCTTGAA TCGCTTCTTC
2101 GTGCTCCGGT TTGAGCGTAA AGGCGGCGAT CGCCTCGACC CTGCGCGCCC TCGAGCTGGG
2161 GGCCGCGATC GAATACGCCC TCTTGCACAT AGACTGAGGC GGTATAATCG TTGTGCGCGG
2221 TATCTGCACT GGGCGGGTGC GAGGCGATAA GTTCATAGAT GGCAAGTTTA TCCTCGATAA
2281 TACGTAAGCG CGCCAGGGGC GAGTTCCGTC ATGTTCTATA CCTCATCTTG CGATGGTGAT
2341 GGCTGACAAA ATTAACGGGA ATCATGATGG TTTATGTTAT GACAGGTAAC AAACCGCTGG
2401 CGATGGCCTG GAAAATCCGT GGCTGAACCC TGGCAATTTC ACATTGATGG TCGCTGGGTC
2461 ACCCTGCCTG TCAGCGGAAA TCTGACCGTG GACGATAGGA CCCGCCTGCT CGCTGCCGCC
2521 GTCGCCGGTG TCGGGGTGGT GCTAGTCACG GAGGAAATGG TGCAAACAGC GCTGCGAAAG
2581 GCAGTCTTTG TCGTCTGTTT GCCGGCAGCG AAGAGACATT CCAGGGCTAC TATCTATGCT
2641 GGCCACAGGG AAAAAGTGAG CTGGTCGCCC ATACCCAGCC GGTGAAAGAA TATATTTATA
2701 TTTTGACGGG CTGTGTGGAT NTCACGGTAN GGGAAACCGT AAGCANGTTA AACGTCCGTG
2761 ATTCGATATT TTATGCCGCC AAAGTCCATA ACCCGGCGCA AGAGGACTGT GAGTTTTTAC
2821 TGGTGATTGA TGGTAAGCAG GCTTGAGGCA GGCTGGCCTT TTTCTACCAG GGCGGACGTC
2881 CCGTTGGACG TCATAGCGGG ATTGGCNCAG GCCATTGTCC CGAATACCCG TAAGCCCAAG
2941 AAGAAACGAT GCCCTTTTTC TGACGTTCGC TCATGGGGAA ATAAACGGAT GAGACCGATA
3001 CGAATGAATT GTTATAAATT TCGCCCAAGG CACAGCAAAA CTTCTATTGA TGGTTAAAGA
3061 TTGCATTGCT TTAAAAAACC GCTTTCTCTA TCATGCATTC TCCTGGTAAA TATAGGCCGA
3121 GAGACTCTCA TGCTATTTAG TCTACATAAT CACATTCTAT GCTCTTCCAT TCCTACCNTA
3181 AGTAATGCCC NTCCCCCTGT TTCTGATGAA ATAAAAATAA AGATCGATAA CATCACCCGG
3241 AATAAACACG TTATGGTATT CAGTGGTTTT TCTTTTCTGG GCTATCATGA CGAACAAGCG
3301 NTTGTCGACA AGTTCAAACA TATTATTTTC CGTGCTATCG TCCAATACGG TATCGGTAAT
3361 TTATGCGTGG GCGCCGGCGC CACCCGTGAA GCTATTGGTA AGGTCTATGA GCTTGCAAAA
3421 GAAAAAGGCA TTCAAACCCT GGGACGGGTG TCGGAACAAG CGCGAGAAAG CACGTCGCTG
3481 TCTGAACATT GTGATGAATG CATTTTCATT TCTGACCCGA

Section eleven

Bibliography

Aksoy, S. (1995a) Molecular analysis of the endosymbionts of tsetse flies: 16S rDNA locus and over-expression of a chaperonin. Insect Molecular Biology. Vol. 4, No. 1, pp 23-29.

Aksoy, S. (1995b) *Wigglesworthia* gen. nov. and *Wigglesworthia glossinidia* sp. nov., taxa consisting of the mycetocyte-associated, primary endosymbionts of tsetse flies. International Journal of Systematic Bacteriology. Vol. 45, No 4, pp 848-851

Aksoy, S., Pourhosseini, A.A. and Chow, A. *(1995) Mycetome* endosymbionts of tsetse flies constitute a distinct lineage related to Enterobacteriaceae. Insect *Molecular* Biology. Vol. 4, No. 1, pp 15-22.

Aldhous, P. (1993) Bacteria may provide access to the tsetse fly. Science. Vol. 261, p548

Altschul S. F., Gish W., Miller W., Myers E. W., Lipman DJ (1990) Basic local alignment search tool. Journal of Molecular Biology. Vol. 215, pp 403-410

Ammer, J., Brennenstuhl, M., Schindler, P., Holtje, J.V. and Zahner, H. (1979) Phosphorylation of streptozotocin during uptake via the phosphoenolpyruvate:sugar phosphotransferase system in *E. coli.* Antimicrobial Agents and Chemotherapy. Vol. 16, pp 801-807.

Anderson, K.L. and Salyers, A.A. (1989) Genetic evidence that outer membrane binding of starch is required for starch utilisation by *Bacteroides thetaiotaomicron.* Journal of Bacteriology. Vol. 171. pp 3199-3204.

Anoxolabehere, D., Kidwell, M.G. and Periquet, G. (1988) Molecular characteristics of diverse populations are consistent with the hypothesis of a recent invasion of *Drosophila melanogaster* by mobile P elements. Molecular Biology of Evolution. Vol. 5, pp 252-269.

Araki, Y. and Ito, Y. (1975) A pathway of chitosan formation in *Mucor rouxii,* Enzymatic deacetylation of chitin. European Journal of Biochemistry. Vol. 55, pp71-78.

Arnold, K. (1992) Chitinolytic activities in nematodes and other parasites: a possible chemotherapeutic target. Ph.D Thesis, Aberdeen University.

Armand, S., Tomita, H., Heyraud, A., Gey, C., Watanabe, T. and Henrissat, B. (1994) Stereochemical course of the hydrolysis reaction catalysed by chitinases A1 and D from *Bacillus circulans* WL-12. FEBS Letters. Vol. 343, pp 177-180.

Ausubel, F.M., Brent, R., Kingston, R., Moore, D.D., Seideman, J.G., Smith, J.A. and Struhl, K. (1990a) Current Protocols in Molecular Biology. Wiley and Sons.

Ausubel, F.M., Brent, R., Kingston, R., Moore, D.D., Seideman, J.G., Smith, J.A. and Struhl, K. (1990b) Short Protocols in Molecular Biology. 2nd edition Wiley and Sons.

Baker, R.D., Maudlin, I., Milligan, P.J.M., Molyneux, D.H. and Welburn, S.C. (1990) The possible role of Rickettsia-like organisms in trypanosomiasis epidemiology. Parasitology. Vol. 100, pp 209-217.

Baldwin, S.A. and Henderson, P.J.,(1989) Homologies between sugar transporters from eukaryotes and prokaryotes. Annual Reviews of Physiology. Vol. 51 pp 459-471.

Barr, A.R. (1966) Cytoplasmic incompatibility as a means of eradication of *Culex pipiens*. Proceedings of the Annual Conference of the Californian Mosquito Control Association. Vol. 34, pp 32-35.

Bassler, B., Gibbons, P. and Roseman, S. (1989) Chemotaxis to chitin oligosacharides by *Vibrio furnissii*, a chitinivorous marine bacterium. Biochemical and Biophysical Research Communications. Vol. 161, No. 3, pp 1172-1176.

Bassler, B.L., Gibbons, P.J., Yu, C. and Roseman, S. (1991a) Chitin utilisation by marine bacteria. Chemotaxis to chitin oligosacharides by *Vibrio furnissii*. The Journal of Biological Chemistry. Vol. 266, No. 36, pp 24268-24275.

Bassler, B.L., Yu, C., Lee, Y.C. and Roseman, S. (1991b) Chitin utilisation by marine bacteria. Degradation and catabolism of chitin oligosacharides by *Vibrio furnissii* Vol. 266, No. 36, pp24276-24286.

Beard, C.B., Mason, P.W,. Aksoy, S., Tesh, R.B. and Richards, F.F. (1992) Transformation of an insect symbiont and expression of a foreign gene in the Chagas disease vector *Rhodnius prolixus*. The American Journal of Tropical Medicine and Hygiene. Vol. 46, pp 195-200.

Beard, C.B., O'Neill, S.L., Tesh, R.B., Richards, F.F. and Aksoy, S. (1993a) Modification of arthropod vector competence via symbiotic bacteria. Parasitology Today. Vol. 9, pp 179-183.

Beard, C.B., O'Neill, S.L., Mason, P., Mandelco, L., Woese, C.R., Tesh, R.B., Richards, F.F. and Askoy, S. (1993b) Genetic transformation and phylogeny of bacterial symbionts from tsetse. Insect Molecular Biology, Vol.1, No 3, pp123-131.

Berger, L.R. and Reynolds, D.M. (1958) The chitinase system of a strain of *Streptomyces griseus*. Biochimica et Biophysica Acta. Vol. 29, pp 522-534.

Bergey's Manual of Systematic Bacteriology (1984) Krieg, N.R. and Holt, J.G. (Ed) Williams and Wilkins, Baltimore, London.

Bergey's Manual of Determinitive Bacteriology. Ninth edition (1994) Holt. J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T. and Williams, S.T. (Ed) Williams and Wilkins, Baltimore, London.

Beyer, M. and Diekmann, H. (1985) The chitinase system in *Streptomyces* sp. ATCC 11238 and its significance for fungal cell wall degradation. Applied Microbiology and Biotechnology. Vol. 23, pp 140-146.

Birnboim, H.C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acid Research. Vol. 7, pp 1513-1523.

Blaak, H., Schnellmann, J., Walter, B., Henrissat, B. and Schrempf, H. (1993) Characteristics of an exochitinase from *Streptomyces olivaceoviridis*, its corresponding gene, putative protein domains and relationship to other chitinases. European Journal of

Biochemistry. Vol. 214, pp 659-669.

Boller, T., Gehri, A., Mauch, F. and Vogeli, U. (1983) Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. Planta. Vol. 157, pp 22-31.

Bonnanfant-Jais, M.L. (1974) Morphologie de la glande lactee d'une glossine, *Glossina austeni* Newst. au cours du cycle de gestation aspects ultrastructuraux en periode de gestation. Journal de Microscopie. Vol. 19, No. 3, pp 265-284.

Boos, W. and Lucht, J.M., (1996) Periplasmic binding protein-dependant ABC transporters. Chapter 76 in *Escherichia coli* and *Salmonella:* Cellular and Molecular Biology. Second edition, Neidhardt, F.C. (Editor in chief) ASM Press Washington.

Bork, P. and Doolittle, R.F. (1992) Proposed acquisition of an animal protein domain by bacteria. Proceedings of the National Academy of Sciences USA. Vol 89. pp 8990-8994.

Brennan, M.D., Rowan, R.G. and Dickenson, W.J. (1984) Introduction of a functional P element into the germ line of *Drosophila hawaiiensis*. Cell. Vol. 38, pp 147-151.

Brewer, J.A.J. and Werren, J.H. (1990) Micro-organisms associated with chromosome destruction and reproductive isolation between two insect species. Nature. Vol. 346, pp 558-560.

Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C.J. and Broflie, R. (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science. Vol. 254, pp 1194-1197.

Brown, T.A. (1991) Molecular Biology Labfax. Blackwell Scientific Publications.

Brurberg, M.B., Eijsink, V.G.H. and Nes, I.F. (1994a) Characterisation of a chitinase gene (*chiA*) from *Serratia marcescens* BJL200 and one step purification of the gene product. Microbiology Letters. Vol. 124, pp 399-404.

Brurberg, M.B., Haandrikmann, A.J., Leenhouts, K.J., Venema, G. and Nes, I.F. (1994b) Expression of a chitinase gene from *Serratia marcescens* in *Lactococcus lactis* and *Lactobacillus plantarum*. Applied Microbiology and Biotechnology. Vol. 42, pp 108-115

Brurberg, M.B., Eijsink, V.G.H., Haandrikman, A.J., Vanema, G. and Nes, I.F. (1995) Chitinase B from *Serratia marcesens* BJL200 is exported to the periplasm without processing. Microbiology. Vol. 141, pp123-131.

Buxton, P.A. (1955) The Natural History of Tsetse Flies. London; H.K. Lewis & Co. Ltd.

Byrne, C.R., Monroe, R.S., Ward, K.A. and Kredlich, N.M., (1988) DNA sequences of the cysK regions of *Salmonella typhimurium* and *Escherichia coli* and linkage of the *cysK* regions to *ptsH*. Journal of Bacteriology. Vol. 170, pp 3150-3157.

Cabib, E. (1987) The synthesis and degradation of chitin. Advances in Enzymology.

Vol. 59, pp 59-101.

Cabib, E., Silverman, S.J. and Shaw, J.A. (1992) Chitinase and chitin synthase: counterbalancing activities in cell separation of *Saccharomyces cerevisiae.* Journal of General Microbiology. Vol. 138, pp 309-316.

Canlas, M., Wadee, A., Lamontagne, L. and Piessens, W.F. (1984) A monoclonal antibody to surface antigens on microfilariae of *Brugia malayi* reduces microfilaremia in infected jirds. American Journal of Tropical Medicine and Hygiene. Vol. 33, pp 420-424.

Chernin, L.S., De la Fuente, L., Sobolev, V., Haran, S., Vorgias, C.E., Oppenheim, A.B. and Chet, I. (1997) Molecular cloning, structural analysis, and expression in *Escherichia coli* of a chitinase gene from *Enterobacter agglomerans.* Applied Environmental Microbiology. Vol. 63, No 3, pp 834-839

Chen J.P., Nagayama, F. and Chung Chang, M. (1991) Cloning and expression of a chitinase gene from *Aeromonas hydrophila* in *Escherichia coli.* Applied and Environmental Microbiology. Vol. 57, No. 8, pp 2426-2428.

Chiang, R.G. and Davey, K.G. (1988) A novel receptor capable of monitoring applied pressure in the abdomen of an insect. Science Vol. 241, pp 1665- 1667.

Chitlaru, E. and Roseman, S. (1996) Molecular cloning and characterisation of a novel β-N-acetyl-D-glucosaminidase from *Vibrio furnissii.* Journal of Biological Chemistry. Vol. 271, pp 33433-33439

Chothia, C. and Lesk, A.M. (1986) The relationship between the divergence of sequence and structure in proteins. EMBO Journal. Vol. 5, pp 823-826.

Chu, G., Hayakawa, H. and Berg, P. (1987) Electroporation for the efficient transfection of mamalian cells with DNA. Nucleic Acids Research. Vol. 15, pp 1311-1326.

Cohen, E. (1987) Chitin biochemistry: synthesis and inhibition. Annual Review of Entomology. Vol. 32, pp 71-93.

Cohen, J.J. (1993) Overview: Mechanisms of apoptosis. Immunology Today. Vol. 14, pp 126-130

Crampton, J., Morris, A., Lycett, G., Warren, A. and Eggleston, P. (1990) Transgenic mosquitoes: a future vector control strategy? Parasitology Today. Vol. 6, no.2, pp 31-36.

Daniels, S.B., Strasbaugh, L.D. and Armstrong, R.A. (1985) Molecular analysis of P element behaviour in *Drosophila simulans* transformants. Molecular and General Genetics. Vol. 200, pp 258-265.

Delic, I., Robbins, P. and Westpheling, J. (1992) Direct repeat sequences are implicated in the regulation of two *Streptomyces* chitinase promoters that are subject to carbon catabolite control. Proceedings of the National Academy of Sciences USA. Vol. 89, pp 1885-1889.

Doyle, J., Hurumi, H., Lupton, N. and Cross, G. (1980) Antigenic variation in clones of animal infective *Trypanosoma brucei* derived and maintained *in vitro*. Parasitology. Vol. 80, pp 359-369.

Durbin, E. and Fallon, A.M. (1985) Transient expression of the chloramphenicol acetyl transferase gene in cultured mosquito cells. Gene. Vol. 36, pp 173-178.

Earhart, C.F. (1996) Uptake and metabolism of iron and molybdenum. Chapter 71 in *Escherichia coli* and *Salmonella:* Cellular and Molecular Biology. Second edition, Neidhardt, F.C. (Editor in chief) ASM Press Washington.

Ellis, D.S. and Evans, D.A. (1977) Passage of *Trypanosoma brucei rhodesiense* through the peritrophic membrane of *Glossina morsitans morsitans*. Nature. Vol. 267, pp 834-835.

Evrall, C.C., Attwell, R.W. and Smith, C.A. (1990) A semi-micro quantitative assay for the determination of chitinolytic activity in microorganisms. Journal of Microbiological Methods. Vol. 12, pp 183-187.

Felgner, P.L., Gadek, T.R. and Holm, M. (1987) A highly efficient lipid mediated DNA transfection procedure. Proceedings of the National Academy of Sciences USA. Vol. 84, pp7413- 7417.

Flach, J., Pilet, P.E. and Jolles, P. (1992) What's new in chitinase research? Experientia. Vol. 48, pp 701-716.

Flyg, C. and Boman, H.G. (1988) *Drosophila* genes *cut* and *minature* are associated with the susceptibility to infection with *Serratia marcescens*. Genetics Research. Vol. 52, pp 51-56.

Frandberg, E. and Schnurer, J. (1994) Evaluation of a chromogenic chito-oligosaccharide analogue, ρ-nitrophenyl-β-D-N,N'-diacetylchitobiose, for the measurement of the chitinolytic activity of bacteria. Journal of Applied Bacteriology. Vol. 76, pp 259-263.

Freeman, J.C. (1973) The penetration of the peritrophic membrane of the tsetse flies by trypanosomes. Acta Tropica. Vol. 30, No. 4, pp 347-354.

Freyvogel, T.A. and Jaquet, C. (1965) The Prerequisites for the Formation of a Peritrophic Membrane in Culicidae Females. Acta Tropica. Vol. 22, pp 148-154.

Fuchs, R.L., McPherson, S.A. and Drahos, D.J. (1986) Cloning of a *Serratia marcescens* gene encoding chitinase. Applied and Environmental Microbiology. Vol. 51, No.3, pp 504-509.

Fuhrman, J.A., Urioste, S.S., Hamill, B., Spielman, A. and Piessens, W.F. (1987) Functional and antigenic maturation of *Brugia malayi* microfilariae. American Journal of Tropical Medicine and Hygiene. Vol. 36, No.1, pp 70-74

Fuhrman, J.A., Lane, W.S., Smith, R.F., Piessens, W.F. and Perler, F.B. (1992) Transmission-blocking antibodies recognise microfilarial chitinase in brugian lymphatic filariasis. Proceedings of the National Academy of Science. Vol. 89, pp 1548-1552.

Fujii, T. and Miyashita, K. (1993) Multiple domain structure in a chitinase gene (*chi*C) of *Streptomyces lividans*. Journal of General Microbiology. Vol. 139, pp 677-686.

Gal, S.W., Choi, J.Y., Kim, C.Y., Cheong, Y.H., Choi, Y.J., Bahk, J.D., Lee, S.Y. and Cho, M.J. (1997) Isolation and characterization of the 54-kDa and 22-kDa chitinase genes of *Serratia marcescens* KCTC2172. FEMS Microbiology Letters. Vol. 151, No 2 pp 197-204

Gilbert, H.J., Hall, J., Hazelwood, G.P. and Ferreira, L.M.A. (1990). The N terminal region of an endoglucanase from *Pseudomonas fluorescens* subspecies *cellulosa* constitutes a cellulose-binding domain that is distinct from the catalytic centre. Molecular Microbiology. Vol. 4, pp 759-767.

Gilboa-Garber, N. and Garber, N. (1989) Microbial lectin cofunction with lytic activities as a model for a general basic lectin role. FEMS Microbiology Reviews. Vol. 63, pp 211-222.

Gimenez, D.F. (1964) Staining rickettsia in yolk-culture. Stain Technology. Vol. 39, pp 135- 140.

Gingrich, J.B., Ward, R.A., Macken, L.M. and Schoenbechler, M.J. (1982). *Trypanosoma brucei rhodesiense* (Trypanosomatidae): factors influencing infection rates of a recent human isolate in the tsetse *Glossina morsitans* (Diptera: Glossinidae). Journal of Medical Entomology. Vol. 19, pp 268-274

Gleave, A.P., Taylor, R.K., Morris, B.A.M. and Greenwood, D.R. (1995) Cloning and sequencing of a gene encoding the 69-kDa extracellular chitinase of *Janthinobacterium lividum*. FEMS Microbiology Letters. Vol. 131, pp 279-288.

Gold, L. and Stormo, G. (1987) Translation initiation pp 1302-1307 in *Escherichia coli* and *Salmonella typhimurium* Cellular and Molecular Biology. Second edition, Neidhardt, F.C. (Editor in chief) ASM Press Washington.

Golic, K.G. and Lindquist, S. (1989) The FLP recombinase of yeast catalyses site-specific recombination in the *Drosophila* genome. Cell. Vol. 59, pp 599-509.

Gooday, G.W. (1990) The ecology of chitin degradation. In: K.C.Marshall (ed) Advances in Microbial Ecology. Vol. pp 387-430. Plenum press, New York.

Gooday, G.W (1994) Physiology of microbial degradation of chitin and chitosan In Biochemistry of Microbial Degradation. Kluwer, C.Ratledge(Ed.), Dordrecht pp 279-312.

Gooday, G.W., Brydon, L.J. and Chappell, L.H. (1988) Chitinase in female *Onchocerca gibsoni* and its inhibition by allosamidin. Molecular and Biochemical Parasitology. Vol. 29, pp 223-225.

Hall, B.G., Imai, K. and Romano, C.P. (1982). Genetics of the Lac-PTS system of *Klebsiella*. Genetics Research. Vol. 39. pp 287-302.

Hanahan, D. (1983) Studies on the transformation of *E.coli* with plasmids. Journal of Molecular Biology. Vol. 45, pp 37-67.

Handler, A.M. and O'Brochta, D.A. (1991) Prospects for gene transformation in insects. Annual Review of Entomology. Vol. 36, pp 159-183.

Hara, S., Yamamura, Y., Fujii, Y., Mega, T. and Ikenaka, T. (1989) Purification and characterisation of chitinase produced *by Streptomyces erythraeus.* Journal of Biochemistry. Vol. 105, pp 484-489.

Harpster, M.H. and Dunsmuir, P. (1989) Nucleotide sequence of the chitinase B gene of *Serratia marcescens* QMB 1466. Nucleic Acids Research. Vol. 17, pp 5395.

Harshbarger, J.C. and Faust, R.M. (1973) Environmental factors internal to the host that affect the success of microbial insecticides. Annals of the New York Academy of Sciences. Vol. 217 pp 130-140

Hart, J.P., Monzingo, A.F., Ready, M.P., Ernst, S.R. and Robertus, J.D. (1993) Crystal structure of an endochitinase from *Hordeum vulgare* L. seeds. Journal of Molecular Biology. Vol. 229, pp 189-193.

Haymer, D.S. and Marsh, J.L. (1986) Germline and somatic instability of a white mutation in *Drosophila mauritiana* due to a transposable element. Developmental Genetics. Vol. 6, pp 281-291.

Henderson, P.J., Baldwin, S.A., Cairns, M.T., Charalambous, B.M., Dent, H.C., Gunn, F., Liang, W.J., Lucas, V.A., Martin, G.E., McDonald, T.P., (1992) Sugar-cation symport systems in bacteria. International Reviews in Cytology. Vol. 137, No,1 pp 49-208

Hengge, R. and Boos, W. (1983). Maltose and lactose transport in *Escherichia coli.* Examples of two different types of concentrative transport systems. Biochimica et Biophysica Acta. Vol. 737. pp 443-478.

Henrissat, B. (1990) Weak sequence homologies among chitinases detected by clustering analysis. Protein Sequences Data Analysis. Vol. 3, pp 523-526.

Henrissat, B. (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochemical Journal. Vol. 280, pp 309-316.

Henrissat, B. and Bairoch, A. (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochemical Journal. Vol. 293, pp 781-788.

Hepburn, H.E. (1985) Structure of the integument. In: Kerkut, G.A. and Gilbert, L.I. (ed) Comprehensive Insect Physiology Biochemistry and Pharmacology. Vol. 3, pp 1-58. Pergamon, Oxford.

Heuber, E. and Davey, K.G. (1974) Bacteroids in the ovaries of a tsetse fly. Nature. Vol. 249, pp 260-261.

Holden, P.R., Jones, P. and Brookfield, J.F.Y. (1996) Evidence for a *Wolbachia* symbiont in *Drosophila melanogaster*. Genetics Research. Vol. 62, pp 23-29.

Hoopes, B.C. and McClure, W.R. (1987) Strategies in regulation of transcription

initiation pp 1231-1240 in *Escherichia coli* and *Salmonella typhimurium* Cellular and Molecular Biology. Second edition, Neidhardt, F.C. (Editor in chief) ASM Press Washington.

Horwitz, M., Reid, J. and Ogaydziak, D. (1984) Genetic improvements of chitinase production of *Serratia marcescens*. In J. Zikakis (Ed) Chitin, Chitosan and Related Enzymes. Academic Press, Orlando. pp 191-208.

Huber, M., Cabib, E. and Miller, L.H. (1991) Malaria parasite chitinase and penetration of the mosquito peritrophic membrane. Proceedings of the National Academy of Sciences USA. Vol. 88, pp 2807-2810.

Huq, A., Small, E.B., West, P.A., Huq, M.I., Rezaur, R. and Colwell, R.R. (1983) Ecological relationships between *Vibrio cholerae* and planktonic crustacean copepods. Applied and Environmental Microbiology. Vol. 45, No. 1, pp 275-283.

Huq, A., West, P.A., Small, E.B., Huq, M.I. and Colwell, R.R. (1984) Influence of water temperature, salinity, and pH on survival and growth of toxigenic *Vibrio cholerae* serovar 01 associated with live copepods in laboratory microcosms. Applied and Environmental Microbiology. Vol. 48, No. 2, pp 420-424.

Ibrahim, E.A.R., Ingram, G.A. and Molyneux, D.H. (1984) Haemagglutinins and parasite agglutinins in haemolymph and gut of *Glossina*. Tropical Medicine and Parasitology. Vol. 35, pp 151-156.

Imoto, T. and Yagishita, K. (1971) A simple activity measurement of lysozyme. Agricultural Biological Chemistry. Vol. 35. No.7, pp 1154-1156.

Inbar, J. and Chet, I. (1991) Evidence that chitinase produced by *Aeromonas cavaie* is involved in the biological control of soil borne plant pathogens by this bacterium. Soil Biology and Biochemistry. Vol. 23, pp 973-978.

I.U.B. (1984) Enzyme nomenclature: Recommendations of the Nomenclature Committee of the International Union of Biochemistry on the Nomenclature and Classification of Enzyme-Catalysed Reactions, Academic Press, London and New York.

Iverson, K.L., Bromel, M.C., Anderson, A.W. and Freeman, T.P. (1984) Bacterial symbionts in the sugar beet root maggot, *Tetanops myopaeformis* (von Roder). Applied and Environmental Microbiology. Vol. 47, pp 22-27.

Izard, J.W. and Kendall, D.A. (1994) Signal peptides: exquisitely designed transport promoters. Molecular Microbiology *Vol. 5 pp* 765-773

Jach, G., Logemann, S., Wolf, G., Oppenheim, A.B., Chet, I., Schell, J. and Logemann, J. (1992) Expression of a bacterial chitinase leads to improved resistance of transgenic tobacco plants against fungal infection. Biopractise. Vol. 1, pp 33-41.

James, A.A. (1992) Mosquito Molecular Genetics: The Hands That Bite Back. Vol. 257, pp 37-38

Jannatipour, M., Soto-Gill, R.W., Childers, L.C. and Zyskind, J.W. (1987) Translocation of *Vibrio harveyi* N,N'-diacetylchitobiase to the outer membrane of

Escherichia coli. Journal of Bacteriology. Vol. 169, No. 8, pp 3785-3791.

Jeuniaux, C. (1966) Chitinases. Methods in Enzymology Neufeld, E.F. and Ginsburg,V.(eds). Vol. 8, pp 644-654.

Johnstone, J. (1908) Conditions of Life in the Sea. Cambridge University Press. pp332.

Jones, J.D.G., Grady, K.L., Suslow, T.V. and Bedbrook, J.R. (1986) Isolation and characterisation of genes encoding two chitinase enzymes from *Serratia marcescens*. EMBO Journal. Vol. 5, No. 3, pp 467-473.

Jones, J.D.G., Dean, C., Gidoni, D., Gilbert, D., Bond-Nutter, D., Nedbrook, J. and Dunsmuir, P. (1988) Expression of a bacterial chitinase protein in tobacco leaves using two photosynthetic gene promoters. Molecular and General Genetics. Vol. 212, pp 536-542.

Jordan, A.M. and Trewern, M.A. (1973) Sub-lethal effect of sulphaquinoxaline on the tsetse fly *Glossina austeni* Newstead. Nature. Vol. 245, p 462.

Jordan, A.M. and Trewern, M.A. (1976) Sulphaquinoxaline in host diet as the cause of reproductive abnormalities in the tsetse fly (*Glossina* spp.). Entomology Experience Applied. Vol. 19, pp 115-129.

Joshi, S., Kozlowski, M., Selvaraj, G., Iyer, V.N. and Davies, R.W. (1988) Cloning of the genes of the chitin utilisation regulon of *Serratia liquefaciens*. Journal of Bacteriology. Vol. 170, No. 7, pp 2984-2988.

Kabelitz, D., Pohl, T and Pechold, K (1993) Activation-induced cell death (apoptosis) of mature peripheral T lymphocytes. Immunology Today. Vol. 14, pp 338-339.

Kamei, K., Yamamura, Y., Hara, S. and Ikenaka, T. (1989) Amino acid sequence of chitinase from *Streptomyces erythraeus*. Journal of Biochemistry. Vol. 105, pp 979-985.

Kaneko, T. and Colwell, R.R. (1973) Ecology of *Vibrio parahaemolyticus* in Chesapeake bay. Journal of Bacteriology. Vol. 113, No. 1, pp 24-32.

Keyhani, N.O., Wang, L., Lee, Y.C. and Roseman, S. (1996) The chitin catabolic cascade in the marine bacterium *Vibrio furnissii*. Characterisation of an N,N'-diacetyl-chitobiose transport system. Journal of Biological Chemistry. Vol. 271, No.52, pp 33409-33413.

Keyhani, N.O. and Roseman, S. (1996a) The chitin catabolic cascade in the marine bacterium *Vibrio furnissii*, Molecular cloning, isolation, and characterisation of a periplasmic chitodextrinase. Journal of Biological Chemistry. Vol. 271, No.52, pp 33414-33424

Keyhani, N.O. and Roseman, S. (1996b) The chitin catabolic cascade in the marine bacterium *Vibrio furnissii*. Molecular cloning, isolation, and characterisation of a periplasmic β-N-acetylglucosaminidase. Journal of Biological Chemistry. Vol. 271, No.52, pp 33425-33432.

Kidwell, M.G., Kidwell, J.F. and Sved, J.A. (1977) Hybrid dysgenesis in *Drosophila*

melanogaster a syndrome of aberrant traits including mutation, sterility, and male recombination. Genetics. Vol. 86, pp 813-833.

Kidwell, M.G., Frydryk, T. and Novy, J.B. (1983) The hybrid dysgenesis potential of *Drosophila melanogaster* strains of diverse temporal and geographic origins. Drosophila Information Service. Vol. 51, pp 97-100

Kidwell, M.G. and Ribeiro, J.M.C. (1992) Can transposable elements be used to drive disease refractory genes into vector populations? Parasitology Today. Vol. 8, pp 325-329.

Kitto, G.B. (1969) Intra- and extramitochondrial malate dehydrogenases from chicken and tuna heart. Methods in Enzymology. Vol. 13, pp 106-116.

Kless, H., Sitrit, Y., Chet, H. and Oppenheim, A.B. (1989) Cloning of the gene coding for the chitobiase of *Serratia marcescens*. Molecular and General Genetics. Vol. 217, pp 471-473.

Kornblihtt, A. R., Umezawa, K., Vide-pederson, K. and Baralle, F.E. (1985) Primary structure of human fibronectin: differential splicing may generate at least 10 polypeptides from a single gene. EMBO Journal. Vol. 4, pp 1755-1759.

Kramer, K.J. and Koga, D. (1986) Insect chitin. Physical state, synthesis, degradation and metabolic regulation. Insect Biochemistry. Vol. 16, No. 6, pp 851-877.

Krasfur, E.S., Whitten, C.J. and Novy, J.E. (1986) Screwworm eradication in north and central America. Parasitology Today. Vol. 3, pp 131-137.

Kuranda, M.J. and Robbins, P.W. (1991) Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. The Journal of Biological Chemistry. Vol. 266. No. 29, pp 19758-19767.

Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. Journal of Molecular Biology. Vol. 157, pp 105-132.
Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. Vol. 227, pp 680-685.

Lamed, R., Naimark, J., Morgenstern, E. and Bayer, E.A. (1987) Specialised cell surface structures in cellulolytic bacteria. Journal of Bacteriology. Vol. 169, pp 3792-3800.

Lamont, G., Tucker, R. and Cross, G. (1986) Analysis of antigen switching rates in *Trypanosoma brucei*. Parasitology. Vol. 92, pp 355-367.

Lancien, J. (1993) La maladie du sommiel conolee au sud de L'ouganda. Actualities. Vol. 41, pp 7-10.

Laven, H. (1967) Eradication of *Culex pipiens fatigans* through cytoplasmic incompatibility. Nature. Vol. 216, pp 383-384.

Lin, T-Y. and Koshland, D.E., Jr. (1969) Carboxyl group modification and the activity of lysozyme. Journal of Biological Chemistry. Vol. 244, pp 505-508.

Lindquist, D.A. (1992) The new world screwworm fly in Libya a review of its introduction and eradication. Medical and Veterinary Entomology. Vol. 6, No. 1, pp 2-8.

Lopata, M.A., Cleveland, D.W. and Sollner-Webb, B. (1984) High level transient expression of a chloramphenicol acetyl transferase gene by DEAE dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. Nucleic Acids Research. Vol. 12 pp 5707-5717.

Lundblad, G., Hedersteadt, J., Lind, J. and Steby, M. (1974) Chitinase in goat serum. Preliminary purification and characterisation. European Journal of Biochemistry. Vol. 46, pp 367-376.

Lund, P. and Dunsmuir, P. (1992) A plant signal sequence enhances the secretion of bacterial ChiA in transgenic tobacco. Plant Molecular Biology. Vol. 18, pp 47- 53.

Lyons, M. (1991) African sleeping sickness: an historical review. International Journal of Sexually Transmitted Diseases and Aids. Vol. 1, No. 2, supplement 1, pp 20-25.

MacFaddin, J. (1976) Biochemical tests for the identification of medical bacteria. Williams and Wilkins Company, Baltimore, USA.

Maeda, H. and Ishida, N., (1967) Specificity of binding of hexopyranosyl polysaccharides with fluorescent brightener. The Journal of Biochemistry (Tokyo). Vol. 62, pp 276- 278.

Maiden, M.C.J., O.Davis, E., Baldwin, S.A., Moore, D.C.M. and Henderson, P.J.F. (1987) Mammalian and bacterial sugar transport proteins are homologous. Nature. Vol. 325 pp 641-643.

Malcolm, B.A., Rosenberg, S., Corry, M.J., Allen, J.S., de Baetselier, A. and Kirsch, J.F. (1989) Site-directed mutagenesis of the catalytic residues Asp-52 and Glu-35 of chicken egg white lysozyme. Proceedings of the National Academy of Sciences USA. Vol. 86, pp133-137

Maloney, P.C. and Wilson, T.H. (1996) Ion-coupled transport and transporters. Chapter 74 in *Escherichia coli* and *Salmonella* Cellular and Molecular Biology. Second edition, Neidhardt, F.C. (Editor in chief) ASM Press Washington.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning A Laboratory Manual. Cold Spring Harbour, New York.

Manson, F.D.C., Fletcher, D.C. and Gooday, G.W. (1992) Distribution of chitinolytic enzymes in blood cells of turbot *Scophthalmus maximus*. Journal of Fish Biology. Vol. 40, pp 919-927.

Martin, M. (1991) The latex of *Hevea brasiliensis* contains high levels of both chitinases and chitinases/lysozymes. Plant Physiology. Vol. 95, pp 469-476.

Maudlin, I. (1991) Transmission of African trypanosomiasis: interactions amoung tsetse immune system, symbionts and parasites. In: Harris, K.F. (ed) Advances in disease vector research. Vol. 7, pp 117-147.

Maudlin, I. and Dukes, P. (1985) Extrachromosomal inheritance of susceptibility to trypanosome infection in tsetse flies. Selection of susceptible and refractory lines of *Glossina morsitans morsitans*. Annals of Tropical Medicine and Parasitology. Vol. 79, pp 317-324.

Maudlin, I. and Ellis, D.S., (1985) Association between intracellular rickettsia like infections of midgut cells and susceptibility to trypanosome infection in *Glossina* spp. Zeitschrift fur Parasitenkunde. Vol. 71, pp 683-687.

Maudlin, I., Dukes, P., Luckins, A.G. and Hudson, K.M. (1986) Extrachromosomal inheritance of susceptibility to trypanosome infection in tsetse flies. Susceptibility of selected lines of *Glossina morsitans morsitans* to different stocks and species of trypanosome. Annals of Tropical Medicine and Parasitology. Vol. 80 pp 97-105.

Maudlin, I. and Welburn, S.C., (1987) Lectin mediated establishment of midgut infections of *Trypanosoma congolense* and *Trypanosoma brucei* in *Glossina morsitans*. Tropical Medicine and Parasitology. Vol. 38, pp 167-170.

Maudlin, I. and Welburn, S.C. (1988) Tsetse immunity and the transmission of trypanosomiasis. Parasitology Today. Vol. 4, no.4, pp 109-111

Maudlin, I. and Welburn, S.C., (1994) Minireview: Maturation of trypanosome infections in tsetse. Experimental Parasitology. Vol. 79, pp 202-205.

McBride, J.D., Stubberfield, C.R. and Hayes, D.J. (1993) Electrophoretic detection of chitinase isoenzymes using the PhastSystem. Electrophoresis. Vol. 14, pp 165-167.

McCreath, K.J. and Gooday, G.W. (1992) A rapid and sensitive microassay for determination of chitinolytic activity. Journal of Microbiological Methods. Vol. 14, pp 229-237.

McGrane, V., Carlson, J.O., Miller, B.R. and Beaty, B.J. (1988) Microinjection of DNA into *Aedes triseratus* ova and detection of integration. American Journal of Tropical Medicine and Hygiene. Vol. 39, pp 502-510.

McNab, R. and Glover, L.A. (1991) Inhibition of *Neurospora crassa* cytosolic chitinase by allosamidin. FEMS Microbiological Letters. Vol. 13, pp 79-82.

Meinke, A., Braun, C., Gilkes, N.R., Kilburn, D.G., Miller, R. jr. and Warren, R.A. (1991) Unusual sequence organisation in CenB, an inverting endoglucanase from *Cellulomonas fimi*. Journal of Bacteriology. Vol. 173, pp 308-314.

Miller, P.M. and Sands, D.C. (1977) Effects of hydrolytic enzymes on plant-parasitic nematodes. Journal of Nematology. Vol. 9, pp 192-197.

Miller, E. and Turner, J. (1981) Analysis of antigenic types appearing in first relapse populations of clones in *Trypanosoma brucei*. Parasitology. Vol. 80. pp 359- 369.

Miller, L.H., Sakai, R.K., Romans, P., Gwadz, R.W., Kantoff, P. and Hayden, G.C. (1987) Stable integration and expression of a bacterial gene in the mosquito *Anopheles gambiae*. Science. Vol. 237, pp 779-781.

Miller, L.H. (1992) The Challenge of Malaria. Science. Vol. 257, pp 36-38

Miller, N. and Lehane, M.J. (1993) Peritrophic membranes, cell surface molecules and parasite tropisms within arthropod vectors. Parasitology Today. Vol. 9, No. 2, pp 45-50.

Mitchell, R. and Alexander, M. (1961) The mycolytic phenomenum and biological control of *Fusarium* in soil. Nature. Vol. 190, pp 109-110.

Miyashita, K., Fujii, T. and Sawada, Y. (1991) Molecular cloning and characterisation of chitinase genes from *Streptomyces lividans* 66. Journal of General Microbiology. Vol. 137, pp 2065-2072.

Molano, J., Duran, A. and Cabib. E. (1977) A rapid and sensitive assay for chitinase using tritiated chitin. Analytical Biochemistry. Vol. 83, pp 648-656.

Monreal, J. and Reese, E.T. (1969) The chitinase of *Serratia marcescens*. Canadian Journal of Microbiology. Vol. 15, pp 689-696.

Montgomery, M.T. and Kirchman, D.L. (1993) Role of chitin binding proteins in the specific attatchment of the marine bacterium *Vibrio harveyi* to chitin. Applied and Environmental Microbiology. Vol. 59, No. 2, pp 373-379.

Morimoto, K., Karita, S., Kimura, T., Sakka, K., Ohmiya, K. (1997) Cloning, sequencing, and expression of the gene encoding *Clostridium paraputrificum* chitinase ChiB and analysis of the functions of novel cadherin-like domains and a chitin-binding domain. Journal of Bacteriology. Vol.179, No 23, pp 7306-7314

Morris, A.C., Eggleston, P. and Crampton, J.M. (1989) Genetic transformation of the mosquito *Aedes aegypti* by microinjection of DNA. Medical and Veterinary Entomology. Vol. 3, pp 1-7.

Morris, A.C., Schaub, T.L. and James, A.A. (1991) FLP-mediated recombination in the vector mosquito *Aedes aegypti.* Nucleic Acids Research. Vol. 19, pp 5895-5900.

Morse, M.L., Hill, K.L., Egan, J.B. and Hengstenberg, W. (1968). Metabolism of lactose by *Staphylococcus aureus* and its genetic basis. Journal of Bacteriology. Vol. 108. pp 690-694.

Murphy, N.B. and Welburn, S.C. (1997) Programmed cell death in procyclic *Trypanosoma brucei rhodesiense* is associated with differential expression of mRNAs. Cell Death and Differentiation. Vol. 4, pp 365-370.

Murray, N.E., Brammer, W. J. and Murray, K. (1977) Lamboid phages that simplify the recovery of *in vitro* recombinants. Molecular and General Genetics. Vol. 150, pp 53-61.

Murray, M., Stear, M.J., Trail, S.C.M., D'ieteran, G.D.M., Agymong, K. and Dwinger, R.H. (1991) Prospects for control. In Trypanosomiasis in Cattle. Owen, J.B. and Axford, R.F.E. (Ed) pp 203-223.

Muzzarelli, R.A.A. (1977) Chitin. Pergamon Press. New York.

Nalin, D.R., Daya, V., Reid, A., Levine, M.M. and Cisneros, L. (1979) Absorbtion and growth of *Vibrio cholerae* on chitin. Infection and Immunity. Vol. 25, No. 2, pp768-770.

Neu, H.C. and Hepple, L.A. (1965) The release of enzymes from *E. coli* by osmotic shock and during the formation of sphaeroplasts. Journal of Biological Chemistry, Vol. 240, No. 9 pp 3685-3692.

Neugebauer, E., Gamuche, B., Dery, C.V. and Brzezinski, R. (1991) Chitinolytic properties of *Streptomyces lividans*. Archives of Microbiology. Vol. 156, pp 164-167.

Neville, A. (1975) Biology of Arthropod Cuticle. Springer Verlag. New York.

Ni, X. and Westpheling, J. (1997) Direct repeat sequences in the *Streptomyces* chitinase-63 promoter direct both glucose repression and chitin induction. Proceedings of the National Academy of Sciences USA. Vol. 94, No 24, pp 13116-13121

Nguyen, J., Francou, F., Virolle, M.J. and Guerineau, M. (1997) Amylase and chitinase genes in *Streptomyces lividans* are regulated by reg1, a pleiotropic regulatory gene Journal of Bacteriology. Vol. 179, No 20, pp 6383-6390.

Nogge, G. (1976) Sterility in tsetse flies (*Glossina morsitans* Westwood) caused by loss of symbionts. Experientia (Basel). Vol. 32, pp 995

Nogge, G. (1978) Aposymbiotic tsetse flies, *Glossina morsitans morsitans* obtained by feeding rabbits immunised specifically with symbionts. Journal of Insect Physiology. Vol. 24, pp 299-304.

Nogge, G. (1980) Elimination of symbionts of tsetse flies (*Glossina m. morsitans* Westwood) by help of specific antibodies. In Schwemmler and Schenk (Editors), Endocytobiology. Endosymbiosis and Cell Biology. A Synthesis of Recent Research. W. deGruyter, Berlin, New York, pp 445-452.

O'Callaghan, C.H., Morris, A., Kirby, S. and Shingler, A.H. (1972) A novel method for the detection of β-lactamases by using a chromogenic cephalosporin substrate. Antimicrobial Agents and Chemotherapy. Vol. 1, pp 283-288.

Oppenheim, A.B. and Chet, I. (1992) Cloned chitinases in fungal plant-pathogen control strategies. Trends in Biotechnology. Vol. 10, pp 392-394.

O'Neill, S.L. and Karr, T.L. (1990) Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. Nature. Vol. 348, pp 178-180.

O'Neill, S.L., Gooding, R.H. and Aksoy, S. (1993) Phylogenetically distant symbiotic microorganisms reside in Glossina midgut and ovary tissues. Medical and Veterinary Entomology. Vol. 7, pp 377-383.

Pearson, W.R. and Lipman, D.J. (1988) Improved tools for biological sequence comparison, Proceedings of the National Academy of Sciences USA. Vol. 85, 2444-2448.

Pegg, G.F. and Young, D.H. (1982) Purification and characterisation of chitinase enzymes from healthy and *Verticillium albo-atrum*-infected tomato plants, and from *V.albo-atrum*. Physiological Plant Pathology. Vol. 21, pp 389-409.

Pell, P.E. and Southern, *D.I. (1975)* Symbionts in the female tsetse fly *Glossina morsitans morsitans*. Experientia (Basel). Vol. 31 pp650-651.

Pell, P.E. and Southern, D.I. (1976) Effect of the coccidiostat sulphaquinoxaline on symbiosis in the tsetse fly, *Glossina* species. Microbiological Letters. Vol. 2 pp 203-211.

Pell, R. and Gottschal, J.C. (1986) Chitinolytic communities from an aerobic estuarine environment. In Muzzarelli, R.A.A., Jeuniaux, C. and Gooday, G.W. Chitin in Nature and Technology. Plenum Press. New York. pp 539-546.

Peri, K.G. and Waygood, E.B. (1988) Sequence of cloned enzyme II $^{N\text{-acetylglucosamine}}$ of the phosphoenolpyruvate: N-acetylglucosamine phosphotransferase system of *Escherichia coli.* Biochemistry. Vol. 27, pp 6054-6061.

Perrakis, A., Wilson, K.S., Chet, I., Oppenheim, A.B. and Vorgias, C.E. (1993) Phylogenic relationships of chitinases. In Chitin Enzymology. Muzzarelli (Ed), pp 217-232.

Perrakis, A., Tews, I., Dauter, Z., Oppenheim, A.B., Chet, I., Wilson, K.S. and Vorgias, E. (1994) Crystal structure of a bacterial chitinase at 2.3 A resolution. Structure. Vol. 2, pp 1169-1180.

Phillips, D.C. (1967) The hen egg white lysozyme molecule. Proceedings of the National Academy of Sciences. USA. Vol. 57, pp 484-495.

Phiri, J. and Ham, P.J. (1990) Enhanced migration of *Brugia pahangi* microfilariae through the mosquito midgut following N-acetylglucosamine ingestion. Transactions of the Royal Society of Tropical and Medical and Hygiene. Vol. 84, p 462.

Pinnock, D.E. and Hess, R.T. (1974) The occurrance of intracellular rickettsia-like organisms in the tsetse flies, *Glossina morsitans, G.fuscipes, G.brevipalpis and G. pallidipes.* Acta Tropica. Vol. 31, pp 70-79

Pires, F.A., Da Silver, J.M. and Teles E Cunha, G. (1950) Posicao actual da tse-tse na area da Sitatonga circunscricao do Mossurize. Estudo comparitivo da infestacao por tripanossomas patogenicos nas *G.morsitans, G.pallidipes, G. brevipalpis e G. austeni,* Mocambique Vol 62 pp 1-59.

Ponnudurai, T., Billingsley, P.F. and Rudin, W. (1988) Differential infectivity of *Plamodium* for mosquitoes. Parasitology Today. Vol. 4, No. 11, pp 319-321.

Postma, P.W. and Lengeler, J.W. (1985) Phosphoenolpyruvate: carbohydrate phosphotransferase system of bacteria. Microbiological Reviews. Vol. 49, No. 3, pp 232-269.

Postma, P.W., Lenegeler, J.W. and Jacobson, G.R. (1996) Phosphoenolpyruvate: Carbohydrate Phosphotransferase Systems. Chapter 75 in *Escherichia coli* and

Salmonella: Cellular and Molecular Biology. Second edition, Neidhardt, F.C. (Editor in chief) ASM Press Washington.

Reinhardt, C., Steiger, R and Heckler, H. (1972) Ultrastructural study of the midgut mycetome-bacteroids of the tsetse flies *Glossina morsitans, G. fuscipes, and G. brevipalpis* (Diptera, Brachycera). Acta Tropica. Vol. 29 pp280-288.

Reyes, F., Calatayud, J., Vazquez, C. and Martinez, M.J. (1989) β-N-Acetylglucosaminidase from *Aspergillus nidulans* which degrades chitin oligomers during autolysis. FEMS Microbiology Letters. Vol. 65, pp 83-88.

Richards, A.G. and Richards, P.A. (1977) The Peritrophic Membrane of Insects. Rockstein, M. (Ed.) Academic Press. pp 205-232.

Richards, F.F. (1993) An approach to reducing arthropod vector competence, Dispersion of insect-borne diseases could be modified by genetically altered symbionts. Science. Vol. 59, no.10, pp 509-514.

Rio, D.C. (1991) Regulation of *Drosophila* P element transposition. Trends in Genetics. Vol. 7, pp 282-287.

Robbins, P.W., Albright, C. and Benfield, B. (1988) Cloning and expression of a *Streptomyces plicatus* chitinase (chitinase-63) in *Escherichia coli.* The Journal of Biological Chemistry. Vol. 263, No. 1, pp 443-447.

Robbins, P.W., Overbye, K., Albright, C., Benfield, B. and Pero, J. (1992) Cloning and high level expression of chitinase encoding gene of *Streptomyces plicatus.* Gene (Amst.). Vol. 111, pp 69-76.

Roberts, R.L. and Cabib, E. (1982) *Serratia marcescens* chitinase: One-step purification and use for the determination of chitin. Analytical Biochemistry. Vol. 127, pp 432-412.

Roberts, W.K. and Selitrennikoff, C.P. (1986) Isolation and partial characterisation of two antifungal proteins from barley. Biochemica et Biophysica Acta. Vol. 280, pp 161-170.

Romaguera, A., Menge, U., Breves, R. and Diekmann,H. (1992) Chitinases of *Streptomyces olivaceoviridis* and significance of processing for multiplicity. Journal of Bacteriology. Vol. 174, No. 11, pp 3450-3454.

Rouboud, E. (1919) Les particularities de la nutrition et la vie symbiotique chez les mouches tsetses. Ann. Inst. Pasteur (Paris). Vol. 33 pp 489-537.

Rouslathi, E. (1988) Fibronectin and its receptors. Annual Review of Biochemistry. Vol 57, pp 375-413.

Rudin, W. and Hecker, H. (1989) Lectin binding sites in the midgut of the mosquitoes *Anopheles stephensi* Liston and *Aedes aegypti* L. (Diptera: Culicidae). Parasitology Research, 75, 268-279.

Rudzinska, M.A., Speilman, A., Lewengrub, S., Piesman, J. and Karakishian, S, (1982)

Penetration of the peritrophic membrane of the tick by *Babesia microti*. Cell Tissue Research. Vol. 221, pp 471-481.

Sacks, D. and Perkins, P.V. (1985) Development of infective stage *Leishmania* promastigotes within phlebotomine sandflies. American Journal of Tropical Medicine and Hygiene. Vol. 34, pp 456-459.

Sadowski, P. (1986) Site specific recombinases: Changing partners and doing the twist. Journal of Bacteriology. Vol. 165, pp 341-347.

Sahai, A.S. and Manocha, M.S. (1993) Chitinases of fungi and plants: their involvement in morphogenesis and host-parasite interaction. FEMS Microbiology Reviews. Vol. 11, pp 317-338.

Saito, T., Suzuki, K., Yamamoto, J., Fukui, T., Miwa, K., Tomita, K., Nakanashi, S., Odani, S., Suzuki, J. I. and Ishikawa, K. (1989) Cloning, nucleotide sequence, and expression in *Escherichia coli* of the gene for poly(3-hydroxybutyrate) depolymerase from *Alcaligenes faecalis*. Journal of Bacteriology. Vol. 171, pp 184-189.

Sanchez-Torres, J., Perez, P. and Santamaria, R.I. (1996) A cellulase gene from a new alkalophilic *Bacillus* sp. (strain N186-1). Its cloning, nucleotide sequence and expression in *Escherichia coli*. Applied Microbiology and Biotechnology, Vol. 2 pp 149-155.

Sanger, F.S., Nicklen, J. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences. USA. Vol. 74, pp 5463-5467.

Sasmal, D., Guhathakurta, B., Ghosh, A.N., Pal, C.R. and Datta, A. (1992) N-acetyl-D-glucosamine-specific lectin purified from *Vibrio cholerae* 01. FEMS Microbiology Letters. Vol. 98, pp 217-224.

Scholz, P., Haring, V., Wittmann-Liebold, B., Ashman, K., Bagdasarian, M. and Scherzinge,r E. (1989) Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. Gene Vol. 75, No 2, pp 271-288

Schlein, Y. (1977) Lethal effect of tetracycline on tsetse flies following elimination of bacteroid symbionts. Experientia. Vol. 33, pp 450-451.

Schlein, Y., Jacobsen, R.L. and Schlomai, J. (1991) Chitinase secreted by *Leishmania* functions in the sandfly vector. Proceedings of the Royal Society of London. Vol. 245, pp 121-126.

Schlein, Y., Jacobsen, R.L. and Messer, G. (1992) *Leishmania* infections damage the feeding mechanism of the sandfly vector and implement parasite transmission by bite. Proceedings of the National Academy of Sciences USA. Vol. 89, pp 9944-9948.

Schlein, Y. (1993) *Leishmania* and sandflies: Interactions in the life cycle and transmission. Parasitology Today. Vol. 9, No. 9, pp 255-258.

Schlein, Y. and Jacobsen, R.L. (1993) Haemoglobin inhibits the development of infective promastigotes and chitinase secretion in *Leishmania major* cultures.

Parasitology. Vol. 109, pp 23-28.

Shahabuddin, M. and Kaslow, D.C. (1993a) Chitinase: a novel target for blocking parasite transmission? Parasitology Today. Vol. 9, No. 7, pp 252-255.

Shahabuddin, M., Toyoshima, T., Aikawa, M. and Kaslow, D.C. (1993b) Transmission blocking activity of a chitinase inhibitor and activation of malarial parasite chitinase by mosquito protease. Proceedings of the National Academy of Sciences USA. Vol. 90, pp 4266-4270.

Shapira, R., Ordentlich, A., Chet, I. and Oppenheim, A.B. (1989) Control of plant diseases by chitinases expressed from cloned DNA in *Escherichia coli*. Phytopathology. Vol. 79, pp 1246-1249.

Shiro, M., Ueda, M., Kawaguchi, and T. Arai, M. (1996) Cloning of a cluster of chitinase genes from *Aeromonas* sp. No 10S-24. Biochimica et Biophysica Acta. Vol. 1305 pp 44-48.

Sieber, K.P., Huber, M., Kaslow, D., Banks, S.M., Torii, M., Aikawa, M. and Miller, L.H. (1991) The peritrophic membrane as a barrier: its penetration by *Plasmodium gallinaceum* and the effect of a monoclonal antibody to ookinetes. Experimental Parasitology. Vol. 72, No. 2, pp 145-156

Silver, S., (1996) Transport of inorgnic cations. Chapter 72 in *Escherichia coli* and *Salmonella:* Cellular and Molecular Biology. Second edition Neidhardt, F.C. (Editor in chief) ASM Press Washington.

Sitrit, Y., Barak, Y., Kapulnik, Y., Oppenheim, A.B. and Chet, I. (1993) Expression of *Serratia marcescens* chitinase gene in *Rhizobium meliloti* during symbiosis on alfalfa roots. Molecular Plant-Microbe Interactions. Vol. 6, No. 3, pp 293-298.

Sitrit, Y., Vorgias, C.E., Chet, I. and Oppenheim, A.B. (1995) Cloning and primary structure of the *chi*A gene from *Aeromonas caviae*. Journal of Bacteriology. Vol. 177, No. 14, pp 4187-4189.

Sneh, B. (1981) Use of rhizospere chitinolytic bacteria for biological control. Phytopath Zeitschrift. Vol. 100, pp 251-256.

Soto-Gil, R.W. and Zyskind, J.W. (1984) Cloning of *Vibrio harveyi* chitinase and chitobiase gene in *Escherichia coli.* In Zikakis, J.P. (ed) Chitin, Chitosan and Related Enzymes. pp 209-223. Academic Press, Orlando.

Soto-Gil, R.W. and Zyskind, J.W. (1989) N,N'-Diacetylchitobiase of *Vibrio harveyi*. Primary structure, processing, and evolutionary relationships. The Journal Of Biological Chemistry. Vol. 264, No. 25, pp 14778-14783.

Spindler-Barth, M. (1993) Hormonal regulation of chitin metabolism in insect cell lines. In Chitin Enzymology. Muzzarelli, R.A.A (Ed) European Chitin Society, Italy. pp 75-82.

Stanley, J., van Slooten, J., Dowling, Finan D.N., T. and Broughton, W.J. (1989) Molecular cloning of the ntrA gene of the broad host-range Rhizobium sp. NGR234, and phenotypes of a site directed mutant. Molecular and General Genetics. Vol. 217,

pp 528-532.

Stevens, L. and Wade, M.J. (1990) Cytoplasmically inherited reproductive incompatibility in *Tribolium* flour beetles: The rate of spread and effect of population size. Genetics. Vol. 124, pp 367-372.

Stiles, J.K., Ingram, G.A., Wallbanks, K.R., Molyneux, D.H., Maudlin, I. and Welburn, S.C. (1990) Identification of midgut trypanolysin and trypanoagglutinin of *Glossina palpalis* spp. (Diptera: Glossinidae). Parasitology. Vol. 101, pp 369-376.

Stuhlmann, F. (1907) Beiträge zur Kenntnis der Tsetsefliege (*Glossina fusca und G. tachinoides*). Arbeitschlag Kunde Gesundhamt Vol. 26, pp 301-383.

Sundheim, L., Poplawsky, A.R. and Ellingboe, A.H. (1988) Molecular cloning of two chitinase genes from *Serratia marcescens* and their expression in *Pseudomonas* species. Physiological and Molecular *Plant Pathology*. Vol. 33, pp 483-491.

Sutherland, D.R., Christensen, B.M. and Lasee, B.A. (1986) Midgut barrier as a possible factor in filarial worm vector competency in *Aedes trivittatus*. Journal of Invertebrate Pathology. Vol. 47, pp 1-7.

Takayanagi, T., Ajisaka, K., Takiguchi, Y. and Shimahara, K. (1991) Isolation and characterisation of thermostable chitinases from *Bacillus licheniformis* X-7U. Biochemica et Biophysica Acta. Vol 1078, pp 404-410.

Takken, W., Oladunmade, M.A., Dengwat, L., Feldman, H., Onah, J.A., Tenabe, S.O. and Hamman, H.J. (1986) The eradication of *Glossina palpalis palpalis* (Robineau-Desvoidy) (Diptera: *Glossinidiae*) using traps, insecticide-impregnated targets and the sterile insect technique in central Nigeria. Bulletin of Entomological Research. Vol 76, pp 275-286.

Taylor, J.L., Jones, J.D.G., Sandler, S., Mueller, G.M., Bedbrook, J. and Dunsmuir, P. (1987) Optimizing the expression of chimeric genes in plant cells. Molecular and General Genetics. Vol. 210, pp 572-577.

Terwisscha van Scheltinga, A.C., Kalk, K.H., Beitema, J.J. and Dijkstra, B.W. (1994) Crystal structures of hevamine, a plant defence protein with chitinase and lysozyme activity, and its complex with an inhibitor. Structure. Vol. 2, pp 1181-1189.

Toleman, M.A. (1993) Characterisation of extrachromosomal replicating DNA elements in Rickettsia-like organisms and construction of a transformation vector. Msc Thesis, University of the West of England.

Tronsmo, A. and Harmaan, G.E. (1993) Detection and quantification of N-acetyl-β-D-glucosaminidase, chitobiosidase and endochitinase in solutions and on gels. Analytical Biochemistry. Vol 208, pp 74-79.

Trudel, J. and Asselin, A. (1989) Detection of chitinase activity after polyacrylamide gel electrophoresis. Analytical Biochemistry. Vol. 178, pp 362-366.

Tsujibo,H., Orikoshi,H., Tanno, H., Fujimoto, K., Miyamoto, K., Imada, C., Okami, Y. and Inamori, Y. (1993a) Cloning, sequence, and expression of a chitinase gene from a marine bacterium, *Alteromonas sp*. Strain 0-7. Journal of Bacteriology. Vol. 175, No.

1, pp 176-181.

Tsujibo, H., Endo, H., Minora, K., Miyamoto, K., Inamori, K., Miyamoto, K. and Inamori, Y. (1993b) Cloning and sequence analysis of the gene encoding a thermostable chitinase from *Streptomyces thermoviolaceus* OPC-520. Gene. Vol. 134. pp 113-117.

Turelli, M. and Hoffman, A.A. (1991) Rapid spread of an inherited incompatibility factor in Californian *Drosophila*. Nature. Vol. 353, pp 440-442.

Ueda, M. and Arai, M. (1992a) Purification and some properties of chitinases from *Aeromonas sp*. no. 10S-24. Bioscience, Biotechnology and Biochemistry. Vol. 56, pp 460-464.

Ueda, M. and Arai, M. (1992b) Purification and some properties of β-N-acetylglucosaminidase from *Aeromonas sp.* no. 10S-24. Bioscience, Biotechnology and Biochemistry. Vol. 56, pp 1204-1207.

Ueda, M., Kawaguchi, T and Arai M. (1994) Molecular cloning and nucleotide sequence of the gene encoding chitinase II from *Aeromonas sp*. No. 10S-24. Journal of Fermentation and Bioengineering. Vol. 78, No. 3, pp 205-211.

Ulhoa, C.J. and Peberdy, J.F. (1991) Regulation of chitin synthesis in *Trichoderma harzianum.* Journal of General Microbiology. Vol. 137, pp 2163-2169.

Usai, T., Matsui, M and Isobe, K. (1990) Enzymatic synthesis of useful chito-oligosacharides utilising transglycosylation by chitinolytic enzymes in buffer containing ammonium sulphate. Carbohydrate Research. Vol. 203, pp 65-77.

Vale, G.A., Bursell, E. and Hargrove, J.W. (1985) Catching out the tsetse fly. Parasitology Today. Vol. 1, pp 106-110.

Van de Ploeg, L., Bernards, A., Rusewijk, F. and Borst, P. (1982) Characterisation of the DNA duplication-transposition that controls the expression of two genes for variant surface glycoproteins in *Trypanosoma brucei*. Nucleic Acids Research. Vol. 10, pp 585-592.

Van Nieuwenhove, S. (1992) Advances in sleeping sickness therapy. Annals de la Societe Belge de Medicine Tropicale. Vol. 41, pp 39-51.

Vassseur, V., Arigoni, F., Anderson, H., Defago, G., Bompeix, G. and Seng, J.M. (1990) Isolation and characterisation of *Aphanocladium album* chitinase-overproducing mutants. Journal of General Microbiology. Vol. 136, pp 2561-2567.

Vickerman, K., Barry, J., Hajduk, J. and Tetley, L. (1980) Antigenic variation in trypanosomes. In The Host Invader Interplay. Elsevier Biomedical Press. pp 170- 190.

Vickerman, K. (1985) Developmental cycles and biology of pathogenic trypanosomes. British Medical Bulletin. Vol. 41, pp 105-113.

Wang, S.L. and Chang, W.T. (1997) Purification and characterization of two bifunctional chitinases/lysozymes extracellularly produced by *Pseudomonas aeruginosa* K-187 in a shrimp and crab shell powder medium. Applied Environmental

Microbiology. Vol. 63, No, 2 pp 380-386

Watanabe, T., Suzuki, K., Oyanagi, W., Ohnishi, K. and Tanaka, H. (1990a) Gene cloning of chitinase A1 from *Bacillus circulans* WL-12 revealed its evolutionary relationship to *Serratia* chitinase and to the type III homology units of fibronectin. Journal of Biological Chemistry. Vol. 265, No. 26, pp 15659- 15665.

Watanabe, T. Oyanagi, W. Suzuki, K. Tanaka, H. (1990b) Chitinase system of *Bacillus circulans* WL-12 and importance of chitinase A1 in chitin degradation. Journal of Bacteriology. Vol. 172, No. 7, pp 4017-4022.

Watanabe, T., Oyanagi, W., Suzuki, K., Ohnishi, K. and Tanaka, H. (1992) Structure of the gene encoding chitinase D of *Bacillus circulans* WL-12 and possible homology of the enzyme to other prokaryotic chitinases and class III plant chitinases. Journal of Bacteriology Vol 174, No.2, pp 408-414.

Watanabe, T., Kobori, K., Miyashita, K., Fujii, T., Sakai, H., Uchida, M. and Tanaka, H. (1993) Identification of Glutamic Acid 204 and Aspartic Acid 200 in Chitinase A1 of *Bacillus circulans* WL-12 as Essential Residues for Chitinase Activity. The Journal of Biological Chemistry. Vol. 268. No. 25, pp 18567-18572.

Watanabe, T., Ito, Y., Yamada, T., Hashimoto, M., Sekine, S. and Tanaka, H. (1994) The Roles of the C-terminal Domain and type III Domains of Chitinase A1 from *Bacillus circulans* WL-12 in chitin degradation. Journal of Bacteriology. Vol. 176, No. 15, pp 4465-4472.

Welburn, S.C., Maudlin, I. and Ellis, D.S. (1987) *In vitro* cultivation of rickettsia-like-organisms from *Glossina* spp. Annals of Tropical Medicine and Parasitology, Vol. 81, No.3, pp 331-335

Welburn, S.C. and Gibson, W.C. (1989) Cloning of a repetitive DNA from the rickettsia-like organisms of tsetse flies (*Glossina* spp.). Parasitology, Vol. 98, pp 81-84.

Welburn, S.C., Maudlin, I. and Ellis, D.S. (1989) Rate of trypanosome killing by lectins in midguts of different species and strains of *Glossina*. Medical and Veterinary Entomology. Vol. 3, pp 77-82.

Welburn, S.C. (1991) The rickettsia-like organisms of *Glossina* spp. Ph.D. Thesis, University of Bristol.

Welburn, S.C. and Maudlin, I. (1991) Rickettsia-like organisms, puparial temperature and susceptibility to trypanosome infection in *Glossina morsitans*. Parasitology. Vol. 102, pp 201-206.

Welburn, S.C. and Maudlin, I. (1992) The nature of the teneral state in *Glossina* and its role in the aquisition of trypanosome infection in tsetse. Annals of Tropical Medicine and Parasitology. Vol. 86, pp 529- 536.

Welburn, S.C., Arnold, K., Maudlin, I. and Gooday, G.W. (1993) Rickettsia-like organisms and chitinase production in relation to transmission of trypanosomes by tsetse flies. Parasitology, Vol. 107, pp 141-145.

Welburn, S.C., Dale, C., Ellis, D., Beecroft, R. and Pearson, T.W. (1996) Apoptosis in procyclic *Trypanosoma brucei rhodesiense in vitro*. Cell Death and Differentiation. Vol.3, pp 229-236.

Wigglesworth, V.B. (1929) Digestion in the tsetse fly: a study of structure and function. Parasitology. Vol. 21 pp 288-321.

Wigler, M., Silverstein, S., Lee, L.S., Fellicer, A., Cheng, X. and Axel, R. (1977) Transfer of purified herpes virus thymidine kinase gene into cultured mouse cells. Cell. Vol. 11, pp 223-232.

Williams, B., Dransfield, R., Brightwell, R. and Rogers, D. (1993) Where are we now? Health Policy and Planning. Vol. 8, pp 85-93.

Wink, M. (1979) The endosymbionts of *Glossina morsitans* and *Glossina palpalis*: cultivation and experiments and some physiological properties. Acta Tropica. Vol. 36, pp215-222.

Wirth, S.J. and Wolf, G.A. (1990) Dye labelled substrates for the assay and detection of chitinase and lysozyme activity. Journal of Microbiological Methods. Vol. 12, pp 197-205.

Worley, K.C., Brent, A., Wiese and Smith, R.F. (1995). BEAUTY: An enhanced BLAST-based search tool that integrates multiple biological information resources into sequence similarity search results. Genome Research Vol. 5 pp 173-184.

Wortman, A.T., Somerville, C.C. and Colwell, R.R. (1986) Chitinase determinants of *Vibrio vulnificus*: gene cloning and applications of a chitinase probe. Applied and Environmental Microbiology. Vol. 52, No. 1, pp 142-145.

Wynne, E.C. and Pemberton, J.M. (1986) Cloning of a gene cluster from *Cellvibrio mixtus* which codes for cellulase, chitinase, amylase and pectinase. Applied and Environmental Microbiology. Vol. 52, No 6, pp 1362-1367.

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. Gene. Vol. 33, pp 103-119.

Yen, J.H. and Barr, A.R. (1973) The etiological agent of cytoplasmic incompatibility in *Culex pipiens*. Journal of Invertebrate Pathology. Vol. 22, pp 242-250.

Yu, C., Lee, A.M. and Roseman, S. (1987) The sugar-specific adhesion/deadhesion apparatus of the marine bacterium *Vibrio furnissii* is a sensorium that continuously monitors nutrient levels in the environment. Biochemical and Biophysical Research Communications. Vol. 149, No. 1, pp 86-92.

Yu, C., Lee, A.M., Bassler, B.L. and Roseman, S. (1991) Chitin utilisation by marine bacteria. A physiological function for bacterial adhesion to immobilised carbohydrates. The Journal of Biological Chemistry. Vol. 266, No. 36, pp 24260-24267.

Zobell, C.E. and Rittenberg, S.C. (1937) The occurrence and characteristics of

chitinoclastic bacteria in the sea. Journal of Bacteriology. Vol. 35, No. 3, pp 275-287.